ROLE OF INTRACELLULAR LABILE ZINC (Zn^{2+}_i) IN LPS-INDUCED APOPTOSIS IN SHEEP PULMONARY ARTERIAL ENDOTHELIAL CELLS (SPAECS)

by

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Kalidasan Thambiayya, PhD

University of Pittsburgh, 2012

We recently noted that exogenous zinc was capable of counteracting LPS-induced decreases in labile $[Zn]_i$ (i.e., TPEN chelatable, FluoZin-3 detectable) and simultaneously abrogated LPS-induced apoptosis in SPAECs. In an abbreviated survey of the effect of LPS on potential zinc transporters, we noted that LPS increased mRNA of zinc importer, SLC39A14 or ZIP14 (and this effect was mimicked by zinc chelator, TPEN) suggesting that increased expression of SLC39A14 may be an homeostatic mechanism to maintain $[Zn]_i$ and reduce cellular toxicity to LPS. In the current study, we noted that knockdown of SLC39A14 with siRNA rendered SPAECs more sensitive to LPS-induced apoptosis and also impaired the ability of exogenous zinc to rescue this effect. We also previously noted that iNOS or chemically derived (S-nitroso-N-acetylpenicillamine (SNAP)) nitric oxide (NO) is associated with resistant phenotype to LPS-induced apoptosis in a zinc dependent fashion. Since ZIP14 expression has been reported to be indirectly upregulated by NO, we pursued this connection in SPAECs by silencing ZIP14 using siRNA technology. We noted that NO-mediated resistance to LPS-induced apoptosis was independent of ZIP14 but was critically dependent upon the presence of sheep metallothionein (MT). In particular, genetic silencing of these collective forms of sheep MT isoforms abrogated the NO-dependent resistant phenotype to LPS-induced apoptosis as well as abolishing NO-mediated increases in $[Zn]_i$. Collectively, these data confirm that increases in labile $[Zn]_i$ are an important component of ZIP14- or NO-mediated resistance to LPS-induced apoptosis.

Cytoprotection via ZIP14 appears to be secondary to transcellular movement of extracellular zinc whereas NO mediated protection is secondary to S-nitrosation of MT and redistribution of intracellular zinc.

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ABBREVIATIONS

ALI Acute lung injury

DMSO dimethylsulfoxide

eNOS endothelial nitric oxide synthase

FBS fetal bovine serum

GSH Glutathione

HBSS Hank's buffered saline solution

HPV Hypoxic pulmonary vasoconstriction

iNOS Inducible nitric oxide synthase

L-NAME L-Nitro-Arginine Methyl Ester

LPS lipopolysaccharide

MLC Myosin light chain

MLCK Myosin light chain kinase

MLCP Myosin light chain phosphatase

MLEC Mouse lung endothelial cells

MRE metal response element

MT Metallothionein

MTF-1 Metal response element-binding transcription factor

NF-κB Nuclear factor-κB

NO Nitric oxide

NO+ Nitrosonium

NOS Nitric oxide synthase

OONO- Peroxynitrite

O^{2-} Superoxide

PKC Protein kinase C

ROCK Rho kinase

ROS Reactive oxygen species

SNO S-nitrosothiols

SPAEC Sheep pulmonary artery endothelial cells

TPEN N,N,N′,N′-Tetrakis(2-pyridylmethyl)-ethylenediamine

Zn^{2+} Zinc

PREFACE

I would like to thank Dr. Bruce Pitt for giving me the opportunity to conduct my graduate work in his lab. I am very grateful to Dr. Pitt for his unconditional help and support through my graduate studies. My graduate training under his mentorship improved my scientific thinking and writing skills. I am grateful for the mentorship he provided.

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1.0 INTRODUCTION

Functional role of intracellular labile zinc in pulmonary endothelium

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1.1 ABSTRACT

After iron, zinc is the most abundant essential trace metal. Intracellular zinc ($[Zn]_i$) is maintained across a wide range of cells and species within a tight range (100 to 500 µM) by a dynamic process of transport, intracellular vesicular storage and binding to a large number of proteins (estimated at 3-10% of human proteome). As such, zinc is an integral component of numerous metalloenzymes, structural proteins, and transcription factors. It is generally assumed that a vanishingly small component of $[Zn]_{i,}$ referred to as free or labile zinc, and operationally defined as the pool sensitive to chelation (by agents such as N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN)) and capable of detection by a variety of chemical and genetic sensors,

participates in signal transduction pathways. Zinc deficiencies, per se, can arise from acquired (e.g., malnutrition, alcoholism) or genetic (e.g., mutations in molecules affecting zinc homeostasis, the informative and first example being acrodermatitis enteropathica) factors or as a component of various diseases (e.g. sickle cell disease, cystic fibrosis, sepsis). Hypozincanemia has profound effects on developing humans and all facets of physiological function (neuronal, endocrine, and immunological) are affected; although considerable less is known regarding cardiovascular pathophysiology. In this review, we provide an update on current knowledge of molecular and cellular aspects of zinc homeostasis and then focus on implications of zinc signaling in pulmonary endothelium as it relates to programmed cell death, altered contractility and septic and aspectic injury to this segment of the lung.

1.2 SIGNIFICANCE

Labile (or free) $[Zn]_i$ is an important effector molecule in signal transduction in many tissues including neurons, immunocytes and epithelium. The molecular determinants of zinc homeostasis and the impact of perturbations in zinc import, transport and binding on cell physiology have recently been described in various extrapulmonary tissues. Relatively, little is known regarding zinc biology in the pulmonary vasculature. Pulmonary endothelium is an important component of pulmonary vasoregulation and also the locus of earliest structural and functional changes in acute lung injury (ALI), insight into zinc homeostasis in this segment of the lung may be important in understanding the etiology of acute and chronic pulmonary

vascular disease. Accordingly, in this report we review the current status of zinc homeostasis in general and summarize recent findings by our laboratory and others regarding perturbations in pulmonary endothelial zinc in health and disease.

1.3 ZINC OVERVIEW

The role of zinc was first reported in 1869 when it was discovered to be important for the growth of *Aspergillus niger* (130). Zinc was not recognized to be important for human life until 1963 when zinc deficiency was discovered as a major contributing factor in nutritional dwarfism syndrome and hypogonadism (129). It is now well established that zinc is important for numerous cellular functions including cell differentiation (128) and division (128, 156), DNA synthesis (127, 156), RNA transcription (127, 156), and maintaining plasma membrane integrity (162). Recent approaches using bioinformatics methods to mine existing protein databases indicate that approximately 10% of the human proteome is zinc dependent (1). Zinc has three major biological roles as a i) structural component of at least 3000 proteins (108), including transcription factors (108), cytokines and receptors (108); ii) catalytic component of more than 300 enzymes (54) that regulate many cellular activities including DNA synthesis and maintaining membrane stability (8, 19, 112); and iii) regulator of enzyme and receptor activity by acting as an activator or inhibitor ion (112). Total intracellular zinc is maintained in a concentration range from 100μM to 500μM (118) across numerous cell types. Zinc is considered a trace metal, however, because more than 99% of intracellular zinc is protein bound. The concentration of labile $[Zn]_i$ is vanishingly small with estimates between 10^{-9} M (31, 95) to 10^{-12}

M (16) and it is this fraction that may act as a second messenger in cell signaling (168, 172) in a fashion well supported for other divalent cations such as calcium.

Zinc has been referred to as a ‘double edged sword’(64) as both zinc deficiency and zinc excess are associated with adverse effects on cell physiology (8, 18, 25, 80, 83, 103, 132). Zinc deficiency stimulates inter-nucleosomal DNA fragmentation and apoptosis in intestinal (47), neural (62), respiratory epithelium (175) and in systemic endothelium (63), and high levels of zinc (>250 μM) are associated with concentration dependent increases in cell death in cultured pulmonary (146, 170) and cerebral (78) endothelia. In contrast, lower zinc concentrations (10μM) (111, 143, 154) have been shown to inhibit cadmium (143)-, linoleic acid (111)-, and tumor necrosis factor-α (TNF-α)- (111) induced apoptosis in systemic endothelial cells. At the systemic level, i) labile $[Zn]_i$ levels were demonstrated to be affected by changes in fluid shear stress levels in mouse aorta and in human umbilical vein endothelial cells indicating that zinc dyshomeostasis in the systemic endothelium may contribute to the development and progression of cardiovascular diseases and ii) zinc supplementation was shown to reverse systemic inflammation and organ damage, with a positive effect on overall mortality in mouse model of sepsis (7). Little is known about the signaling role of labile $[Zn]_i$ in the pulmonary endothelium in the context of lung diseases. In this review, we discuss the impact of zinc homeostasis and signaling, as well as its efficacy as a cyto-protectant in pathophysiological processes of pulmonary endothelial cell injury and death.

1.3.1 Zinc homeostasis in the cell

Intracellular zinc concentration is maintained by the coordinated activity of a large family of zinc transporters (ZnT & ZIP) (161) and zinc binding proteins such as metallothionein (MT) (161) (Figure 1)**.** Zinc transporters are encoded by one of two of the solute-linked carrier (SLC) gene families: SLC 30 (also known as zinc exporters or ZnT1-10) (13) and SLC39 (also known as zinc importers or ZIP1-14) (13)**.** ZnT transporters reduce cytoplasmic zinc by promoting zinc efflux from cells or into intracellular vesicles, while ZIP transporters increase cytoplasmic zinc by promoting zinc influx from extracellular and, perhaps, from vesicular stores into cytoplasm(100).

1.3.2 Metallothionein

MT: Metallothioneins are major zinc binding proteins that dynamically coordinate up to 7 mol Zn^{2+}/mol MT via cysteine residues (approximately mol 30%) (9). MT is involved in: (i) detoxification of heavy metals like mercury, cadmium, and alkylating cancer drugs (65, 91); (ii) scavenging free radicals (65); and (iii) protection against DNA damage (65), oxidative stress (65), and apoptosis (45). Mammals express at least four isoforms - MT-1, MT-2, MT-3 and MT-4. In humans, there are at least 16 MT genes located in chromosome 16 and most of them are associated with the MT-1 isoform (36). MT-1 and MT-2 are expressed in many tissues and are particularly abundant in the liver, pancreas, intestine and kidney (116). MT-3 and MT-4 are minor isoforms with specific expression patterns in brain (MT-3) and stratified squamous epithelial cells (MT-4) (24). At the subcellular level, MT can be localized to a number of cellular compartments (i.e., mitochondria,

cytosol and nucleus) (173) as well as in the extracellular space (61). The reduction potential of MT (less than -366mV (9)), makes it highly sensitive to physiological oxidants. We (121) and others (85, 86, 104, 107) have shown that MT is sensitive to changes in cellular redox state and demonstrated that increases in reactive oxygen (109) or nitrogen (99, 178) intermediates can oxidize or transnitrosate cysteines in its zinc sulfur clusters leading to liberation of zinc. As such, MT can be viewed as acting as a sensor and switch and connecting changes in cellular redox status with alterations in labile zinc (figure 1).

1.3.3 Solute-linked carrier (SLC) gene families: SLC 39 (or ZIP)

ZIPs: Fourteen ZIP family members have been reported in mammals (46, 75, 96). The majority of ZIP family members are located on the plasma membrane (41, 43, 52, 151, 152, 155, 166) with the exception of ZIP7-8 and ZIP13 (figure 1), which are present in intracellular organelles**.** Gene knock-out technologies have provided valuable information regarding biological significance of the ZIP family members. Knockout (KO) mice lacking ZIP1, ZIP2, and ZIP3 are reported to have abnormal embryogenesis under zinc-limiting conditions (41, 42, 123). ZIP4 KO mice embryos die during early development, whereas heterozygous mice exhibit a phenotype similar to acrodermatitits enteropathica (AE) secondary to impairment of intestinal absorption of zinc (44, 89, 165). ZIP13 KO mice suffer from disorganization in hard connective tissue, including bone, teeth, skin and eyes (49). In humans, lack of ZIP13 is associated with spondylocheiro dysplasia, a form of Ehlers-Danlos syndrome (49, 56). Mice lacking ZIP14 have impaired G-protein coupled receptor (GPCR) signaling (51) and exhibit retarded growth and impaired gluconeogenesis (see table 1).

1.3.4 Solute-linked carrier (SLC) gene families: SLC 30 (or ZnT)

ZnTs: Ten ZnT family members have been reported in mammals (96). Most ZnTs are located on intracellular organelles (i.e., golgi, endosomes and endoplasmic reticulum) (33) (figure 1). ZnT 1 is the only ZnT exporter located at the plasma membrane, compatible with its role as the primary regulator of cellular zinc efflux (120). ZnT1 knockout mice are embryonic lethal (22). Disruption of the *ZnT* genes yields diverse phenotypes providing insight into the biologic function and specificity of the various family members. Mutations in ZnT2 (27) and ZnT4 (71) result in the production of zinc deficient milk in women and mice, respectively. ZnT3 knockout mice are prone to seizures (22). Mice lacking in ZnT5 show growth retardation and osteogenic problems (73) and exhibit impaired cytokine production in mast cells (117). Single–nucleotide polymorphism (SNPs) in ZnT8 are associated with type 2 diabetes in humans (134), and deletion of the ZnT8 gene results in impaired insulin secretion in mice (126)(see table 1).

Table 1. Phenotypical profile (in mouse, *human, ** drosophila, * sheep) of mutants of zinc transporters**

Gene name	Protein name	Type	Phenotypes
SLC39A1	ZIP1	-/-	Abnormal embryogenesis (41, 42)
SLC39A2	ZIP2	-/-	Abnormal embryogenesis (123)
SLC39A3	ZIP3	-/-	Abnormal embryogenesis (41, 42)
SLC39A4	ZIP4	-/-	Death of embryos during development (44)
SLC39A4	ZIP4*	+/-	Inherited disorder acrodermatitits enteropathica (AE), in which the intestines ability to absorb zinc is impaired (89, 165)
SLC39A6	ZIP6	+/-	** Abnormal gonad formation (110) and E-cadherin expression (163)
SLC39A7	ZIP7	+/-	Impaired melanin synthesis (139)
SLC39A8	ZIP8	+/-	Resistance to cadmium-induced testicular damage (35)
SLC39A13	ZIP13	-/-	a) Disorganization in hard connective issue, including bone, teeth, skin and eyes (49) b) * Spondylocheiro dysplatic, a form of Ehlers- Danlos syndrome (14, 35)
SLC39A14	ZIP14	-/-	Impaired GPCR signaling. Growth retardation and impaired gluconeogensis (67)
SLC39A14	ZIP14	KD	a)***Blocked extracellular zinc-mediated protection against apoptosis in SPAECs (154) b)*** Lowered base level zinc in SPAECs (154)
SLC30A1	ZnT1	-/-	Embryonic lethal (3)
SLC30A2	ZnT2	+/-	* Zinc deficiency in milk (27)
SLC30A3	ZnT3	-/-	Seize (22, 30)
SLC30A4	ZnT4	-/-	Zinc deficiency in milk (71)

Table 1 (continued)

SLC30A5	ZnT5	-/-	(a) Growth retardation and osteogenic problem (73) (b) Impaired cytokine production in mast cells (117)
SLC30A7	ZnT7	-/-	Growth retardation, low body zinc status and low fat accumulation (72)
SLC30A8	ZnT8	-/- SNPs	Impaired insulin secretion (126) * Type 2 diabetes (134)

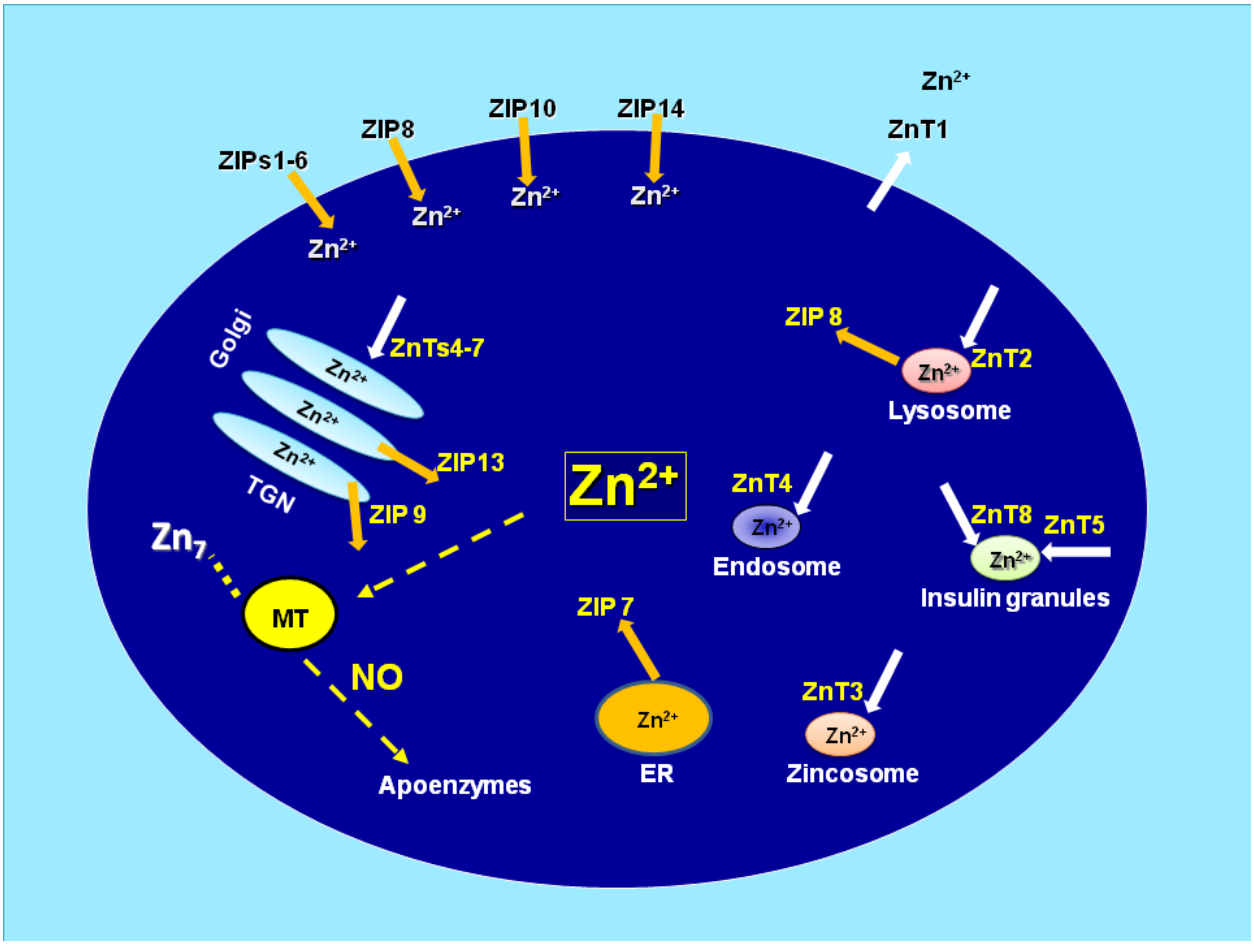

Figure 1. Subcellular localization of zinc transporters and metallothionein

Figure 1. The labile pool of intracellular zinc is tightly controlled by zinc importers (ZIPs), zinc exporters (ZnTs), zinc storing vesicles and zinc binding proteins such as metallothionein (MT). MT plays a critical role in zinc homeostasis acting as a buffer in the steady state while controlling the cellular distribution of transiently elevated zinc in response to perturbations and/or agonists such as nitric oxide (NO)(106). *Modified figure from references (50, 51, 114).*

Manipulation of intracellular zinc levels have been shown to influence the expression and localization of zinc transporters (96) with reports of increased expression of members of ZIP

family and decreased expression of ZnT family members in response to decreases in intracellular zinc (17, 37, 90, 119, 158). Most of these studies have been performed in intestinal and respiratory epithelial or immune cells. While reported increases in ZIP1 and ZIP14 (90)(90) mRNA were shown to be normalized by dietary zinc supplementation in a mouse model of acute lung inflammation (90), the mechanisms underlying the association between zinc homeostasis and lung disease remain largely unknown. ZIP6 was shown to play a role in blocking LPS-induced decreases in intracellular labile zinc and consecutive maturation in mouse dendritic cells (81). Zinc mediated cytoprotection against TNF-α-induced damage in human lung epithelial cells was shown to be dependent upon expression ZIP8 (13). We recently reported (154) in cultured sheep pulmonary artery endothelial cells (SPAECs) that i) ZIP14 is sensitive to changes in intracellular labile zinc and ii) exogenous zinc mediated protection against LPS-induced apoptosis is dependent upon ZIP14.

1.4 ZINC HOMEOSTASIS IN THE PULMONARY ENDOTHELIUM

The Zalewski laboratory in Adelaide, Australia were the first to image labile zinc in the airway (19, 159) and provide evidence that zinc chelation (via TPEN) enhanced hydrogen peroxide-induced caspase activation (159). As reviewed by Troung Tran et al (156), intracellular zinc has also shown to be important for ciliary function, wound healing (via re-epithelialization) and suppression of oxidative stress and apoptosis in the airway epithelium. Further evidence suggests that zinc deficiency sensitizes the lung to acute lung injury following i) alcohol induced epithelial dysfunction (74); ii) hyperoxia (148); and iii) polymicrobial sepsis (7, 82). We have

shown that zinc chelation (via TPEN) exacerbates LPS-induced apoptosis in pulmonary endothelium (SPAECs) (146). TPEN also reversed the protective effect of nitric oxide (NO) donors on LPS-induced apoptosis (147). More recently, we reported that LPS induced time-dependent decreases in intracellular labile zinc (figure 2) in SPAECs using both live cell imaging and fluorescence-activated cell sorting (FACS) with the zinc-sensitive fluorophore, FluoZin-3 (Life Technologies, Grand Island, NY) (153). We further verified the observed decrease in FluoZin-3 detectable zinc using a chimeric reporter encoding a zinc-sensitive metal-response element (MRE) fused to a luciferase gene (153). The LPS-induced changes in labile zinc were accompanied by increases in ZIP14 mRNA. These effects were blocked by addition of exogenous zinc, as was LPS-induced apoptosis (increased caspase 3/7 activity and PS externalization) (153). In separate studies in SPAEC, siRNA knockdown of ZIP14 decreased basal levels of intracellular labile zinc and blocked zinc uptake (as determined by FluoZin-3), and abrogated zinc mediated protection against LPS-induced apoptosis (observed in WT and scrambled control) (154). Collectively, these data suggest that endogenous levels of labile zinc can modulate the sensitivity of pulmonary endothelium to the proapoptotic effects of LPS (figure 2) and implicate ZIP14 in affecting the ability of extracellular zinc to inhibit LPS-induced apoptosis in SPAEC (figure 3).

The results we obtained in pulmonary arterial endothelial cells isolated from mature sheep are distinct from those obtained in SPAEC from fetal sheep. We initially noted that addition of large concentrations of zinc to the medium of SPAEC was associated with necrosis (146). In contrast elevations in intracellular labile zinc (via addition of exogenous zinc (170) or after exposure to large doses of H_2O_2 (169) or NO (170)) were reported to induce apoptosis in

fetal SPAEC. Alternatively, we have consistently noted that chelation of intracellular zinc with TPEN led to dose-dependent apoptosis in mature SPACE whereas a similar maneuver inhibited apoptosis in fetal SPAEC (169, 170).

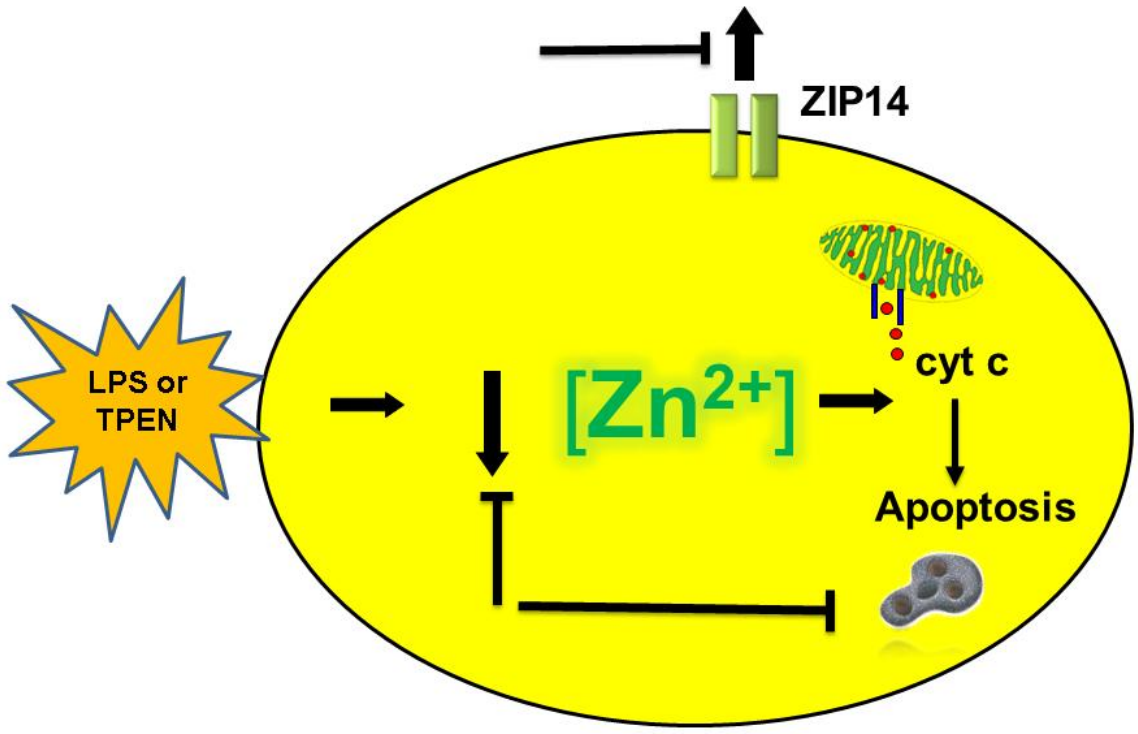

Figure 2. Functional role of labile zinc in LPS-induced apoptosis

Figure 2. LPS caused a decrease in labile zinc in SPAECs (as determined by zinc indicator, FluoZin-3, activity of zinc-sensitive MRE, and changes in steady-state mRNA of zinc importer, ZIP14). The contributory role of decreases in labile zinc in LPS-induced apoptosis (as determined by caspase-3/7 activation, cytochrome c release, and PS externalization) was verified

by mimicking the effects of LPS with zinc chelator, TPEN. Blocking LPS- or TPEN- induced decreases in labile zinc inhibited consecutive increase in apoptosis and ZIP14 mRNA providing support for a signaling role of labile zinc in pulmonary endothelium.

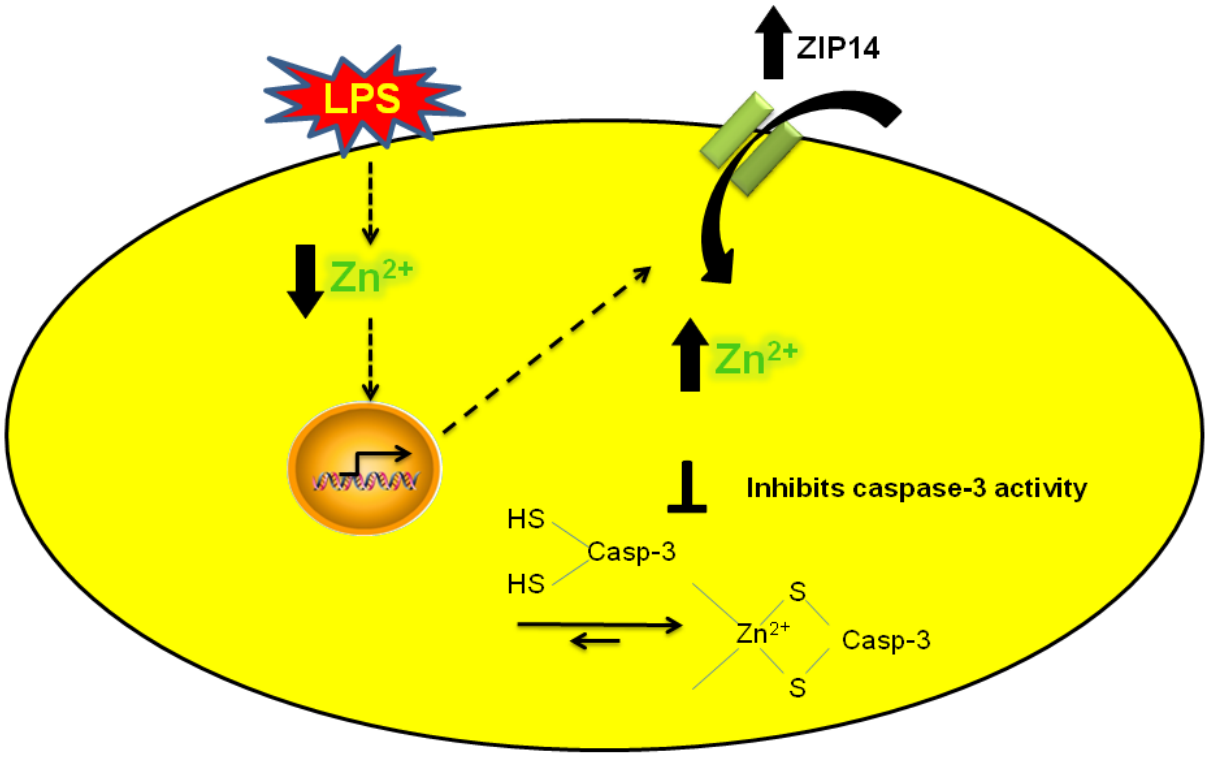

Figure 3. Cytoprotective effect of exogenous zinc is ZIP14 dependent

Figure 3. LPS induced decreases in labile zinc are associated with increases in capsase-3 activity and upregulation of zinc importer, ZIP14 to restore the loss of labile zinc mediated by LPS. Elevation in labile zinc via ZIP14 inhibits apoptosis by inhibiting caspase-3 activity. siRNA to

ZIP14 blocked zinc uptake and abrogated zinc mediated protection against LPS-induced apoptosis.

1.4.1 NO-(MT)-Zn^{2+} signaling in pulmonary endothelium

NO can S-nitrosate metallothionein (88) and cause the release of Zinquin detectable changes in labile zinc in intact cells (10). We (121, 138) have confirmed these observations and demonstrated that: i) S-nitrosation caused conformational changes of MT (via fluorescence resonance energy transfer techniques) in intact pulmonary endothelium consistent with zinc release (121, 137); ii) NO caused an increase in labile zinc in pulmonary artery endothelial cells (138); and iii) MT was the requisite target for NO resulting in such changes in labile zinc (138). Subsequent investigations supported the potential for MT to participate in intracellular signal transduction pathways in pulmonary endothelium.

1. Exposure of mouse lung endothelial cells (MLEC) to the NO donor, S-nitroso-N-acetylpenicillamine (SNAP, 200 μM), caused nuclear translocation of the zinc dependent transcription factor, MTF-1 and such activation was not apparent in MT null cells. Translocation of MTF-1 was associated with NO mediated increase in MT gene expression itself (140) suggested that S-nitrosation of zinc-thiolate clusters in MT and subsequent alterations in zinc homeostasis are participants in intracellular NO signaling pathways affecting gene expression.

2. We observed that zinc chelation (TPEN) abrogated hypoxic vasoconstriction in isolated perfused mouse lungs (IPL), and that IPL from MT null mice showed significantly less constriction than wild-type controls. Data obtained using NO-sensitive FRET reporters supported both enhanced NO production and S-nitrosation of MT during hypoxic exposure. These events were accompanied by NO-dependent increases in labile zinc (Fluo-Zin-3) in subpleural vessels of MT +/+, but not MT -/- mice. These data supported a role for zinc thiolate signaling in pulmonary vasoregulation. Subsequent studies in cell-based models revealed a link between hypoxia induced elevations in labile zinc and changes in myosin light chain phosphatase (MLCP) activity, ultimately leading to stress fiber formation and endothelial cell contraction (11) (figure 5).

3. Most recently, we showed that zinc chelation abrogates NO-mediated protection against LPS-induced apoptosis (154). Relative changes in labile zinc after exposure to cytoprotective doses of the NO donor SNAP (250μM) or exogenous zinc (10μM) were assessed by Fluozin-3, and a comparable increase in intracellular labile zinc was noted in both conditions (154). We further showed that both NO-mediated increases in labile zinc, and NO-mediated protection against LPS-induced apoptosis are dependent on MT via siRNA to sheep MT isoforms (154), thus implicating NO-MT-Zn^{2+} signaling in apoptotic pathways in the pulmonary endothelium (figure 4).

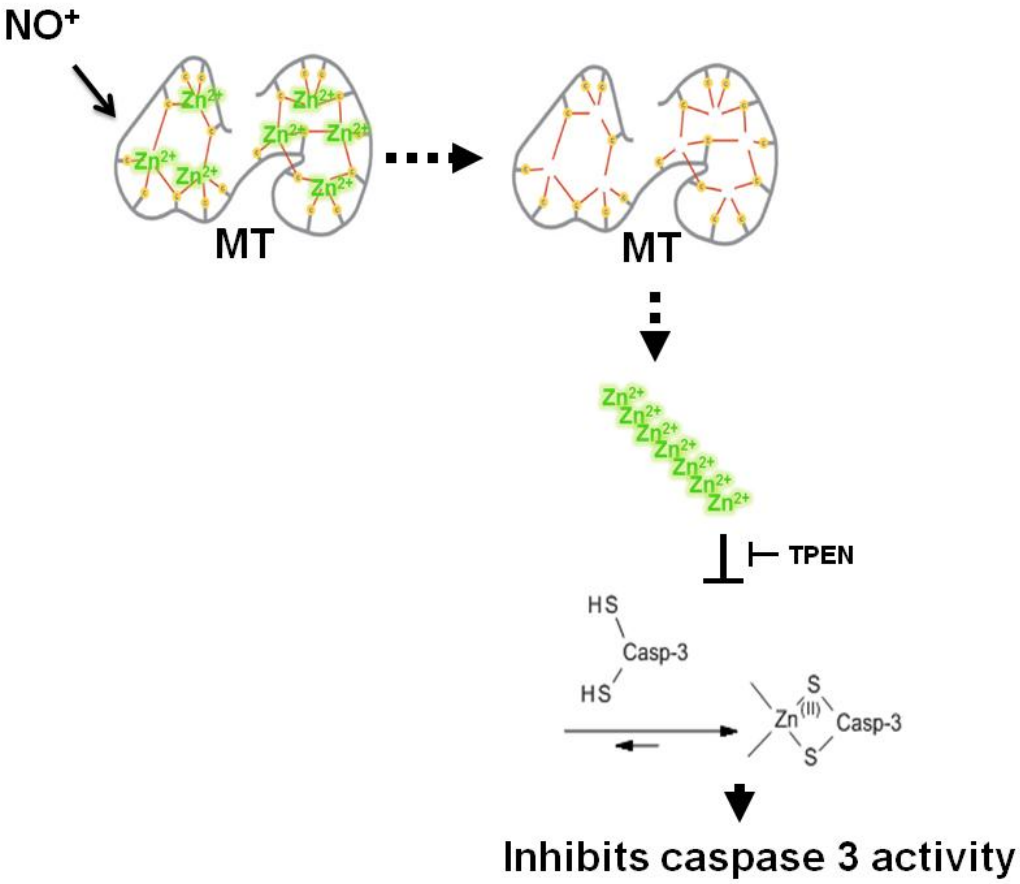

Figure 4. NO elevated zinc from MT inhibits apoptosis in pulmonary endothelial cells

Figure 4. Illustrates the link between apoptosis and elevation in labile zinc (via NO-MT signaling), which in turn inhibits capsase-3 activity in pulmonary endothelial cells. *Modified figure from references (9, 154).*

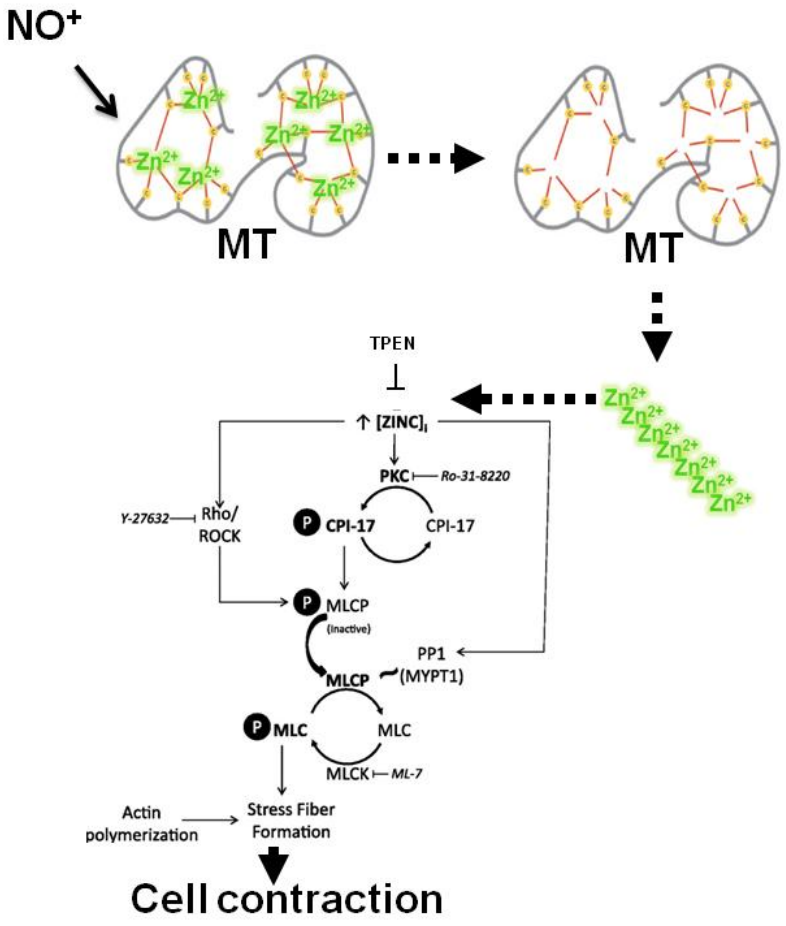

Figure 5. NO elevated zinc from MT causes pulmonary endothelial contraction in hypoxia

Figure 5. Illustrates the link between hypoxia, elevation in labile zinc (via NO-MT signaling), and activation of PKC, which in turns acts via CPI-17 to prevent MLCP activation and promote MLC phosphorylation, ultimately inducing stress fiber formation and pulmonary endothelial cell contraction. *Modified figure from references (9, 11).*

1.4.2 Zinc as an effector molecule in pulmonary endothelium

Decreases in labile zinc have been reported to precede the earliest detectable alterations in cell function (40), morphology (40), and apoptosis (40, 153). We and others have reported that chelation of zinc causes spontaneous apoptosis in pulmonary endothelia (146) and epithelia (156) and elevations in labile zinc via ZIP14 (154) or iNOS induced NO or NO donors (20, 32, 135, 136, 153, 160) inhibits apoptosis in cultured pulmonary endothelial cells. An anti-apoptotic role of zinc has been reported in relation to a variety of stimuli including TNF-α (131), cadmium (142), cholesterol (102) and linoleic acid (111) induced apoptosis. Although the molecular mechanism by which zinc inhibits apoptosis is not clear several reports suggest that zinc inhibits: i) Ca^{2+}/Mg^{2+}-dependent endonucleases that are responsible for DNA fragmentation (15); ii) the activity of caspase-3, a critical protease in apoptosis (122); iii) the processing of caspase-3 (21, 113); and iv) bax activation, cytochrome c release, and apoptosome function (167). Zinc also increases the ratio of Bcl-2 to Bax resulting in the inhibition of caspase activity (176). We reported in SPAECs that: i) decreases in labile zinc mediated by LPS causes casapse-3 activation (153); ii) LPS-induced caspase-3 activity is sensitive to pan caspase inhibitor (153); and iii) extracellular zinc inhibits LPS-induced caspase-3 activity (153, 154). We posed a question whether zinc directly binds capsase-3 and modulates its activity. Our results in-vitro confirmed that zinc directly inhibits caspase-3 activity (154). Although NO can S-nitrosate caspase-3 and inhibit its activity (94), our results suggest that s-nitrosation of MT by NO leads to a release of zinc that is associated with a TPEN dependent cytoprotective caspase-3 inhibition, leading us to suggest that direct S-nitrosation of caspase-3 alone is not likely to account for these results. Collectively, our observation adds to the elegant studies in airway epithelium (19, 159) that revealed: i) labile zinc proximity with procaspase-3 prevents the activation of procaspase-3 and

ii) zinc depletion activates procaspase-3 (159). These studies provide support for the antiapoptotic role of labile zinc in the lung.

1.4.3 Zinc homeostasis and acute lung injury: complexities of integrated response

Several studies have demonstrated that zinc deficiency sensitizes the lung to acute injury. In particular, dietary restriction led to enhanced sensitivity to polymicrobial sepsis (7, 82). Hyperoxic (149, 150) lung injury in mice and macrophage and epithelial cell dysfunction in alcohol fed rats was ascribed to zinc deficiency (74). Zinc repletion reversed phenotype in all three conditions. Although pulmonary endothelial cell dysfunction may have been a component of all these models, any supportive insight into the cellular contributions of zinc dyshomeostasis to these observations largely relates to background information on zinc in respiratory epithelium.

Nonetheless, hyopzincanemia in septic or aseptic (33, 96) conditions is a somewhat underappreciated phenomenon. Transmigration of zinc from tissues, including lung, to liver has been noted in hyperoxia (92), bacterial sepsis (124) and turpentine injury (101) and has been presumed to subserve: i) gluconeogenesis in liver; ii) new protein synthesis in acute phase response; or iii) host defense in an analogous fashion to hypoferronemia in bacterial pneumonia (101). Hepatic expression of ZIP14 appears critical in this pheonenom (97). We recently (unpublished observations) noted that hepatic expression of metallothionein was important in transmigration of zinc from lung to liver during hyperoxic lung injury apparently contributing to the unexpected observation that MT null mice were resistant to hyperoxia. Collectively, these observations suggest that additional insight into the mechanisms underlying such transmigration

may provide new therapeutic targets and strategies and potentially support exogenous zinc as a rational therapeutic agent in acute lung injury.

In summary, compelling evidence is emerging in pulmonary endothelium to complement a larger and growing body of experience in extrapulmonary tissue that labile zinc is a key effector molecule. Critical aspects of the magnitude of labile pool of intracellular zinc accounting for these signaling pathways awaits more refined ratiometric or quantitative fluorescent indicators. Genetic and acquired aspects of zinc dyshomeostasis and deficiencies await further insight into the function and cellular distribution of large family of zinc transporters and metal binding proteins. Nonetheless, it is apparent that the facile and common nature of zinc and nitric oxide chemistry support a role for NO-MT-Zn^{2+} pathway and the uqibuitous nature of these molecules in sepsis and acute lung injury make them a rational novel therapeutic target.

1.5 HYPOSTHESIS AND SPECIFIC AIMS

The functional role of labile zinc has been characterized in neurons, systemic endothelia and immune cells in health and diseases. However, little is known about the functional role of labile zinc in the pulmonary endothelium. These hypotheses will determine the functional role of labile [Zn_i] in pulmonary endothelium in the context of LPS-induced apoptosis.

Specific Aim IA: To determine the functional role of changes in [Zn_i] in SPAEC by LPS stimulation. We hypothesize that LPS results in a decrease in intracellular labile zinc and this decrease is a critical intracellular signaling component transducing the apoptotic effects of LPS.

Specific Aim IB: To determine the functional role of zinc importer, ZIP14, in LPS signaling in SPAECs. We hypothesize that LPS upregulates zinc importer, ZIP14 and elevates labile zinc, and elevation in labile zinc is an important contributing factor that inhibits LPS-induced apoptosis.

Specific Aim II: To determine whether nitric oxide (NO) induced elevation of zinc from metallothionein (MT) protects cells from the LPS. We hypothesize that the release of zinc from MT by NO is an important contributing factor that inhibits LPS-induced apoptosis.

The publications herein (chapters 3 & 4) will discuss our recent findings that delineate labile zinc as a signaling molecule and cytoprotectant in LPS-induced pulmonary endothelial apoptosis.

2.0 MATERIALS AND METHODS

2.1 ISOLATION AND CULTURE OF SPAECS

SPAECs were cultured from sheep pulmonary arteries obtained from a nearby slaughterhouse as previously described (137). Early passage cells were sorted to homogeneity based on uptake of fluorescent labeled di-LDL and subcultures routinely monitored for PECAM (CD31) expression to assure endothelial phenotype and purity. The SPAECs were grown in OptiMEM (GIBCO) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere with 5% CO_2.

2.2 REAGENTS

N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), LPS (0111:B4), active recombinant caspase-3 enzyme, and zinc chloride were purchased from Sigma-Aldrich (St. Louis, MO) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) was purchased from Molecular Probes (Eugene, OR). Caspase-3 substrate Ac-DEVD-AMC (AMC = 7-Amino-4-methylcoumarin) was purchased from Enzo Life Science (Plymouth Meeting, PA).

2.3 FLUORESCENCE MICROSCOPY

SPAECs were cultured on Lab-Tek™ chambered 1.0 borosilicate coverglass slides (Naperville, IL). Cells were washed (2X) with HBSS (Ca^{2+}/Mg^{2+}) and incubated (37 °C; 20 minutes) with 5 µM FluoZin-3 AM (Molecular Probes, Eugene, OR) and equal volume of Pluronic F-127 (Invitrogen, Carlsbad, CA) in HBSS (Ca^{2+}/Mg^{2+}). Subsequently, cells were washed (2X) with HBSS (Ca^{2+}/Mg^{2+}) and immediately imaged in the presence of HBSS (Ca^{2+}/Mg^{2+}) before and after SNAP (2mM) exposure (10 minutes). All recordings were performed at room temperature (20-25 °C). Cells were imaged using a Nikon TE2000E equipped with a 40X 1.3NA oil-immersion objective, Lambda DG4 wavelength switcher, and xenon light source (Sutter Instrument, Novato, CA), charge-couple device camera (Cool-SNAP HQ, Photometrics, Tucson, AZ), and NIS-Elements software (Nikon, Melville, N.Y). FluoZin-3 was excited at 488 nm and emission was detected using a 505 nm to 550 nm bandpass filter. Cells were randomly selected and the mean fluorescence intensity was quantified from the region of interest (ROI) of all randomly selected cells. Background subtraction was performed on all images prior to quantitation.

2.4 FLOW CYTOMETRY

Quantification of relative changes in [Zn_i] was also performed via flow cytometry (115). SPAECs were incubated (37 ^{0}C; 20 min) with 2-5 μM FluoZin-3 AM ester (Molecular Probes) with Pluronic F-127 (equal volume) (Invitrogen) in HBSS (Ca^{2+}/Mg^{2+}) (Invitrogen). LPS treated cells were rinsed in PBS, trypsinized, and centrifuged at 1500 rpm for 5 min. The cell pellet was resuspended with PBS containing 100 μg/ml propidium iodide and incubated (37°C; 15 minutes) in the dark. In a separate series of experiments, phosphatidylserine (PS) externalization was determined with an annexin V–FITC apoptosis detection kit (Biovision, Mountain View, CA). LPS treated cells were rinsed in PBS, trypsinized, and centrifuged at 1500 rpm for 5 min. The cell pellet was resuspended in 300μl binding buffer and supplemented with 3μl of FITC-Annexin-V and 3μl of PI, and incubated (RT; 15 minutes) in the dark. In some experiments, cells were treated with pan-caspase inhibitor Z-VAD-FMK (Calbiochem®, Gibbstown, NJ) at 30μM for 1 h and then treated with LPS (100 ng/ml) or buffer (control). Flow cytometric analysis was performed using a FACSCanto (BD Biosciences, San Jose, CA). For each sample 10,000 events were recorded and analyzed.

2.5 TRANSIENT TRANSFECTIONS AND LUCIFERASE ASSAYS

SPAECs (80-90% confluence) were transfected with 0.6 μg of a luciferase reporter construct driven by 4 metal response element (MRE) tandem repeats (pLuc-MCS/MRE) and 0.15 ug of pSVβ-galactosidase containing *E. coli* lac Z gene reporter construct (Promega, Madison, WI) using Lipofectamine and PLUS reagents (Invitrogen) (115). Cells were lysed and luciferase assay was performed using the Luciferase Assay System (Promega). Relative light units (RLU) were determined in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). β-galactosidase assay was performed using β-galactosidase Enzymes Assay System (Promega). Absorbance was read at 420 nm with a plate reader (Perkin–Elmer, Waltham, MA). Results were expressed as a ratio of firefly luciferase activity to β-galactosidase activity.

2.6 ACCUMULATION OF CYTOCHROME C (CYT C) IN CYTOSOL

Translocation of cyt c was examined by Western blot. After LPS (100 ng/ml) treatment, SPAECs were harvested and resuspended in lysis buffer containing 250 mM sucrose, 20 mM HEPES-potassium hydroxide(pH 7.5), 10 mMpotassium chloride, 1.5 mM magnesium chloride, 1 mM EDTA , 1 mM EGTA, 1 mM dithiothrestol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/mlaprotinin, 1 mg/mlleupeptin, and 0.05% digitonin for 3 min on ice, then centrifuged at 8,500 × g for 5 min. The resulting cytosolic supernatants were subjected to 12% SDS-PAGEand transferred to a nitrocellulose membrane thatwas probed with mouse antibodies against cyt c (BD Pharmingen™,San Diego,CA) or β-actin (Sigma-Aldrich), followed by horseradish peroxidase–coupled detection.

2.7 CASPASE 3/7 ASSAY

SPAECs were seeded on a 96 well (BD Falcon™ white/clear bottom) plate. After treatment, cells were incubated (RT; 1 h) with luminescence Caspase-Glo® 3/7 substrate (Promega). Luminescence was measured using a Fusion-α plate reader (Perkin–Elmer).

2.8 VIABILITY ASSAY

In addition to FACS analysis of PI, viability of SPAECs was determined by quantifying reduction of a fluorogenic indicator Alamar Blue (Biosource, Camarillo, CA). Oxidized Alamar Blue is taken up by cells andreduced by intracellular dehydrogenases.and Thewater-soluble changes in fluorescence emission (590 nm) are utilized as an index of viability (44).

2.9 RNA ISOLATION AND REAL-TIME RT-PCR

Total RNA was isolated using RNAqueous®-4PCR Kit (Ambion, Austin, TX) from untreated and treated SPAECs. The RNA extract was treated with DNase I (Ambion) to remove genomic DNA contamination and quantified by measuring absorbance (260 nm). Total RNA was reverse transcribed using iScript™, (Biorad, Hercules, CA) according to manufacturer's instructions. The cDNA was amplified by real-time PCR using TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA).

2.10 PRIMER DESIGN

Primer Design and PCR amplification efficiency. For most zinc importers and transporters gene, ovine sequences are not available but these genes are conserved across bovine and human species (34). Bovine zinc importers and transporters gene sequences were blasted against the ovine genome using ovine genome browser version 1.0 (livestockgenomics.csiro.au) and primers were designed from bovine sequence (based on conserved regions) using PrimeTime qPCR (IDT DNA technologies, Coralville, IA). Housekeeping primers glucose-6-phosphate dehydrogenase (G6PD) and target primers MT-I,a, -Ib, -Ic and -II were designed from ovine sequence (NCBI database) using PrimeTime qPCR (Integrated DNA Technologies). Amplification efficiency (E) was calculated for each gene from the slope of the dependence of amplification cycle vs. RNA concentration after running serial dilutions of RNA using the formula $E = [10^{(-1/slope)}-1]\times 100$.

2.11 siRNA TRANSFECTION

Silencer® select scramble siRNA and Silencer® select siRNA constructs for targeted genes (MT-Ia,-Ib,-Ic, -II, and ZIP14) were purchased from Ambion. The MT-Ia,-Ib and -Ic genes are highly conserved. We designed one siRNA construct to target against these three genes based on their conserved regions. Separate siRNA constructs were used for other targeted genes. Lipofectamine^{TM} RNAiMAX transfection reagent (Invitrogen) was used to transfect siRNA into SPAECs according to manufacturer's instructions. Briefly, Lipofectamine^{TM} RNAiMAX plus siRNA were added into each well containing OptiMEM. After incubation (20 minutes; RT),

medium containing cells was added to each well. Cells were transfected with siRNA for 72 h. To confirm knockdown, mRNA levels were determined by qPCR.

2.12 RECOMBINANT CASPASE-3 ASSAY

The buffer containing caspase-3 enzyme was replaced with Chelex 100 treated 10mM sodium phosphate buffer (pH 7.4) containing DTT (100 M). 18 nM (final concentration) of caspase-3 enzyme and 50 µM (final concentration) of substrate (DEVD-AMC) was added to the reaction buffer containing 20 mM HEPES in the presence or absence of indicated amount of zinc. Fluorescence (excitation wavelength, 380 nm; emission wavelength, 440 nm) of liberated AMC was measured with spectrophotofluorimeter following incubation for the indicated duration at RT.

2.13 STATISTICAL ANALYSIS

Data are expressed as mean ± SE. Statistical analysis was performed using one-way or two-way analysis of variance (ANOVA) with post hoc comparisons to determine whether the mean of each treatment is different from the untreated cells (control). $p < 0.05$ was considered statistically significant. All statistics were performed using GraphPad Prism version 5 (GraphPad software, San Diego, CA).

3.0 LPS-INDUCED DECREASE IN INTRACELLULAR LABILE ZINC [ZN_I] CONTRIBUTES TO APOPTOSIS IN CULTURED SHEEP PULMONARY ARTERY ENDOTHELIAL CELLS (SPAECS)

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3.1 ABSTRACT

A role in signal transduction for a vanishingly small labile pool of intracellular zinc ([Zn_i]) has been inferred by the sensitivity of various physiological pathways to zinc chelators such as N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) and/or associations with changes in non-protein bound zinc sensitive fluorophores. Although we (146) reported that LPS induced apoptosis in cultured sheep pulmonary artery endothelial cells (SPAEC) was exacerbated by TPEN, (a) we did not detect acute (30 min) changes in [Zn_i]; and (b) it is unclear from other reports whether LPS increases or decreases [Zn_i] and whether elevations or decreases in [Zn_i] are associated with cell death and/or apoptosis. In the current study, we used both chemical (FluoZin-3 via live cell EPI fluorescence microscopy and FACS) and genetic (luciferase activity of a chimeric reporter encoding zinc sensitive metal response element and changes in steady state mRNA of zinc importer (SLC39A14 or ZIP14)) techniques to show that LPS caused a delayed time dependent (2-4 h) decrease in [Zn_i] in SPAEC. A contributory role of decreases in [Zn_i] in LPS-induced apoptosis (as determined by caspase 3/7 activation, annexin-V binding and cytochrome c release) in SPAECs was revealed by mimicking the effect of LPS with the zinc chelator, TPEN and inhibiting LPS- (or TPEN)-induced apoptosis with exogenous zinc. Collectively, these are the first data demonstrating a signaling role for decrease in [Zn_i] in pulmonary endothelial cells and suggest that endogenous levels of labile zinc may affect sensitivity of pulmonary endothelium to the important and complex pro-apoptotic stimulus of LPS.

3.2 INTRODUCTION

Intracellular zinc ([Zn_i]) is maintained within a narrow range (100 to 500 μM) across numerous cell types and species by the coordinate activity of a large family of Zn importers (SLC39A1-14 or ZIP1-14) and transporters (SLC30A1-10 or ZnT1-10), intracellular storage vesicles and Zn-binding proteins (33, 46). Like iron (and other divalent cationic metals), it is considered a trace element because its labile concentration is vanishingly small with estimates between10^{-9} M (31, 95) to 10^{-12} M (16). Depending upon the species, zinc is associated with 3-10% of the genome (2) and in humans is an essential component of more than 300 enzymes, 2000 transcription factors and a large number of receptors, cytoskeletal proteins and other potential regulatory targets (114). As such, the labile pool of [Zn_i] has been considered in the context of intracellular signal transduction including pathways of cell growth, death and differentiation and inflammation, contraction and secretion. Although difficult to quantify, this labile pool is operationally defined as the intracellular zinc compartment chelated by molecules such as N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) and/or detected by non-protein bound zinc sensitive fluorophores (including FluoZin-3).

In contrast to the role of zinc in the central nervous, immune, reproductive, gastrointestinal and/or endocrine systems, considerably less is known about zinc in the lung. It is noteworthy that zinc deficiency (via dietary manipulations) in experimental animals exacerbates lung injury secondary to hyperoxia (148), cecal ligation and puncture (82) and alcohol (74). In an analogous fashion to calcium, however, most studies assessing a role for [Zn_i] in pulmonary endothelium (and other organs and cell types) have focused on transient elevations in [Zn_i] and associations with peroxide induced cell death (146, 170) and contraction (12). A recent report

(81), however, noted for the first time that a decrease in [Zn_i] may be a critical signaling component in the context of LPS-induced maturation of cultured mouse dendritic cells. We (146) noted that TPEN caused a dose-dependent increase in spontaneous apoptosis in pulmonary endothelium and TPEN exacerbated LPS-induced apoptosis in cultured sheep pulmonary artery endothelial cells (SPAEC); a phenomenon noted by others (111) in cytokine and lipid induced apoptosis in cultured systemic endothelium. Nonetheless, in our original study (146), we were not able to detect LPS-induced acute (30 min) changes in [Zn_i]. Others (58) have reported that LPS actually acutely increases [Zn_i] in human leukocytes and that hydrogen peroxide induced increases in [Zn_i] are associated with apoptosis in fetal sheep pulmonary artery endothelial cells (169). Accordingly, we sought to determine if LPS-induced changes in [Zn_i] occurred somewhat later (2-4 h) after exposure of SPAEC to a proapoptotic stimulus of LPS (68-70) and whether changes in [Zn_i] were necessary and sufficient to mediate LPS-induced apoptosis in SPAEC. As decreases in trace metals can be subtle and subject to artifact, we utilized multiple chemical (live cell fluorescence; FACS) and genetic (chimeric reporter encoding zinc sensitive region of metal responsive element fused to luciferase; steady state mRNA levels of ZIP14) detection systems. The ability of TPEN to mimic the effect of LPS-induced apoptosis and rescue of LPS phenotype with exogenous zinc are consistent with a central role for decreases in [Zn_i] and LPS induced apoptosis in SPAEC.

3.3 RESULTS

3.3.1 LPS causes a decrease in labile [Zn_i] as measured by microspectrofluorimetry in live SPAECs.

SPAECs were exposed to LPS or TPEN for 4 h and monitored for changes in FluoZin-3 fluorescence intensity as an index of labile zinc using live cell epifluorescence microscopy. A typical example of such images at 4 h post treatment is shown in Fig1. Compared to control (Fig 6A), LPS decreased overall fluorescence as shown in Fig 6B. As previously noted (12), FluoZin-3 reports labile or TPEN sensitive zinc (Fig 6C). SPAECs were exposed to LPS for up to 4 h and relative changes in mean fluorescence intensity of FluoZin-3 revealed a time-dependent decrease in [Zn_i] as shown in Fig 6D. There was no significant change in overall relative fluorescence at either 0.5 or 1.0 h post LPS and then a significant decrease in [Zn_i] was observed at 2 and 4 h of LPS treatment (Fig. 6D, 330-400 cells per time point from 5 independent experiments).

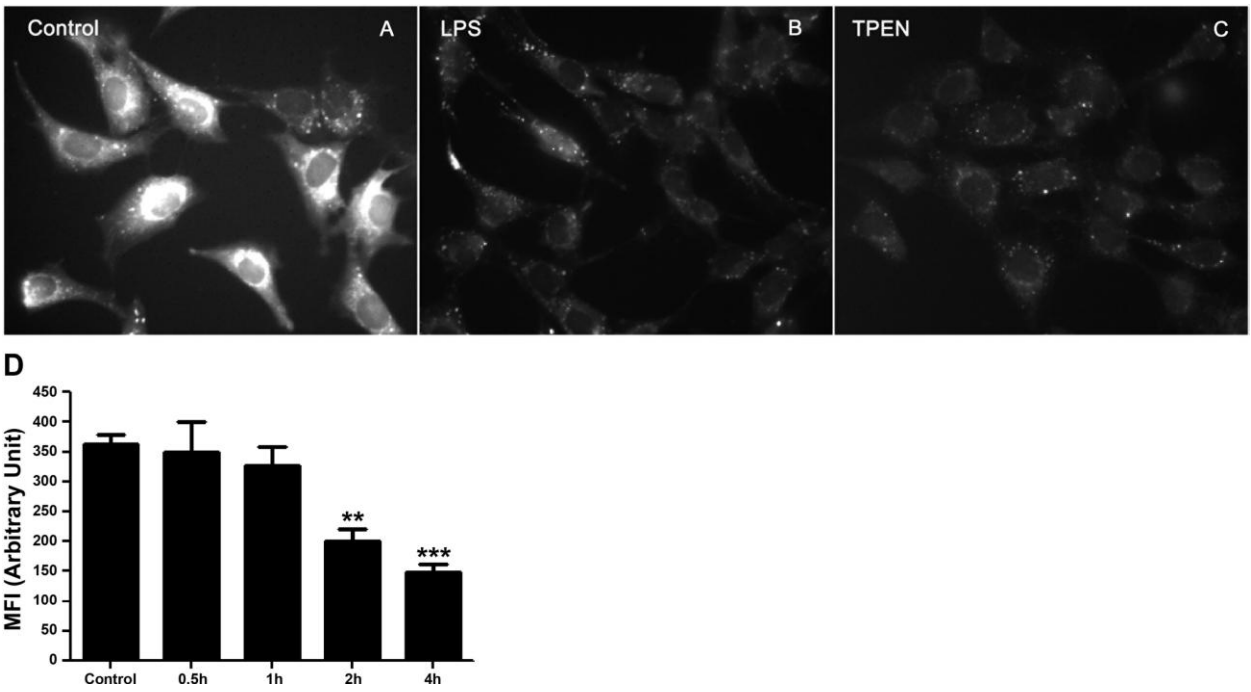

Figure 6. Effect of LPS on labile [Zn_i] in live SPAEC as determined by microspectrofluorimetry

Figure 6. SPAECs were treated with (B) LPS (100ng/ml) or (C) TPEN (2µM) for 4 h. Cells were loaded with 5 µM FluoZin-3 AM and equal volume of Pluronic F-127 and imaged by epifluorescence microscope. The images represent fluorescence intensity of FluoZin-3-Zn complex in SPAECs. All images were captured with identical gain, 100% light intensity, 1 millisecond light exposure and 4x4 binning. (D) Time dependent LPS-induced changes in FluoZin-3 fluorescence in live SPAEC. SPAECs were treated with HBSS (Ca^{2+}/Mg^{2+}) in the presence of LPS (100ng/ml) for 30 minutes, 1 h, 2 h and 4 h respectively. Control cells received HBSS (Ca^{2+}/Mg^{2+}) in the absence of LPS for 4 h. The data represents mean ± SEM of mean fluorescence intensity (MFI) of 330-400 randomly selected cells from 5 experiments for each time point. Images were captured using identical gain and camera settings. For analysis of

images, background illumination was subtracted from the readings. ** and *** designates $p < 0.01$ and $p < 0.001$ respectively, compared to control; one-way ANOVA-Tukey).

3.3.2 LPS causes a decrease in labile [Zn_i] as determined by flow cytometry

To confirm LPS induced changes in [Zn_i] in larger sample size, we used FACS to determine relative changes in FluoZin-3 fluorescence for 10,000 cells at each time point (0.5 to 4 h) on three different occasions. Representative histograms of cell number vs relative fluorescence are shown for one such subculture of SPAEC treated with LPS (Fig 7A). The mean relative fluorescent intensity (MFI) is calculated for the three experiments at each time point, and reported in Fig 7B. In agreement with the imaging data shown in Fig1, the FACS data (Fig 7A) showed a time-dependent leftward shift in MFI indicative of an LPS-induced decrease in [Zn_i]. During the 4 h period of this experiment, cell viability was > 93% (data not shown) as ascertained by propidium iodide in either control or LPS treated SPAEC. Although it appears that a somewhat earlier decrease in [Zn_i] was apparent by FACS (Fig 7B) vs live cell imaging (Fig 6D), an additional time delay in FACS (e.g. trypsinizing, centrifugation, injection on FACS) makes direct temporal comparison between the two methods challenging.

Figure 7A

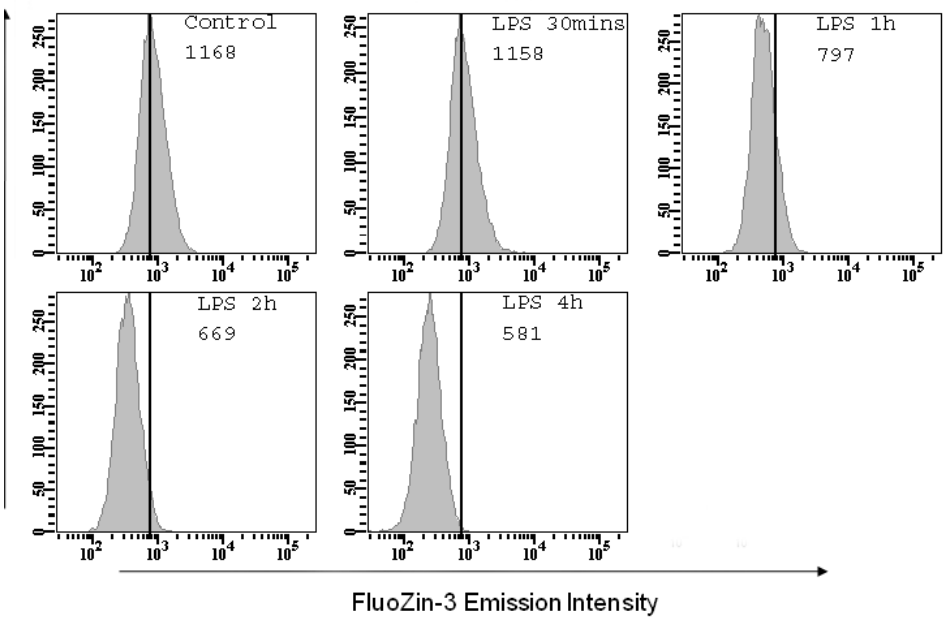

Figure 7B

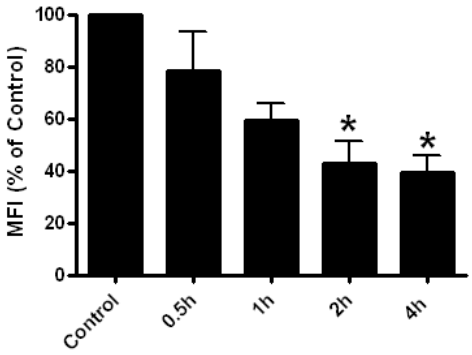

Figure 7. LPS causes a decrease in labile [Zn_i] as determined by flow cytometry

Figure 7. (A) Typical histograms of subcultures of SPAEC treated with LPS for up to 4 hrs. SPAECs were treated with HBSS (Ca^{2+}/Mg^{2+}) in the presence of LPS (100ng/ml) for 30 min, 1 h, 2 h and 4 h respectively. Control cells received HBSS (Ca^{2+}/Mg^{2+}) in the absence of LPS for 4 h. Data demonstrates the histogram of mean relative fluorescence intensity (MFI) of 10000 cells for each time point. The histograms are representative of one subculture of SPAEC treated with LPS. Numbers in each histogram indicates MFI. (B) The bar graph represents mean ± SEM of MFI (% of control) of samples measured in triplicates for 3 independent experiments. * designates $p < 0.01$ compared to control; one-way ANOVA-Tukey).

3.3.3 LPS decreased labile [Zn_i], as revealed by activity of a zinc-sensitive genetically encoded chimeric reporter.

As an alternative to fluorescence detection of changes in [Zn_i], we used a genetic approach and monitored the activity of a highly selective zinc sensitive chimera (23). SPAECs were transiently cotransfected with pLuc-MCS/MRE and pSVβ-galactosidase. The former plasmid expresses the reporter gene luciferase under the control of tandem repeats of the exclusively zinc sensitive metal responsive elements (MRE). Beta-galactosidase activity was measured to account for any differences in transfection efficiency. As shown in Fig 8A, there was a significant decrease in relative luciferase activity in SPAEC exposed to LPS for 4 h. As was noted above in fluorescence based experiments, cell viability (as determined by Alamar Blue) was > 92% in cells transfected with above plasmids with or without addition of LPS (Fig 8B).

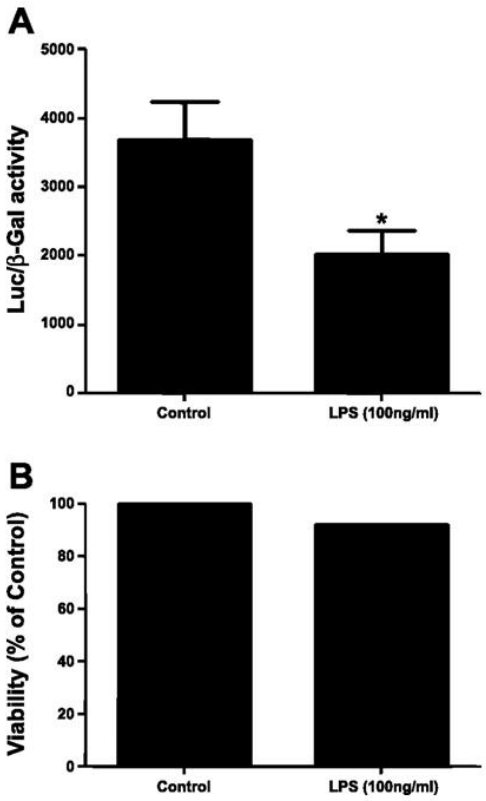

Figure 8. LPS decreases expression of a chimeric zinc sensitive reporter.

Figure 8. (A) SPAECs were transiently cotransfected with pLuc-MCS/4MREa (0.6ug) and pSVβ-galactosidase (0.15ug). 24 h after transfection, cells were treated with or without LPS (100ng/ml) for 4 h in the presence of serum (10%) and subsequently assayed for luciferase and β-galactosidase activity 4 h post treatment. A significant decrease in luciferase activity was observed in LPS stimulated cells with viability greater than 92%. Luciferase activity is expressed as a ratio of firefly luciferase (pLucMRE) activity to β-galactosidase activity. Data represents mean ± SEM of luciferase activity of samples measured in triplicates for 9 independent experiments (* designates $p < 0.05$; independent, two-tailed t-test). (B) Cell viability was assessed by Alamar Blue. Alamar Blue fluorescence was measured as an index of viability 4 h

post LPS treatment. Data represents Alamar Blue fluorescence (% of control) of 9 samples measured in triplicates.

3.3.4 LPS (and decreases in labile [Zn_i]) upregulates SLC39A14 (ZIP14) mRNA expression

We surveyed the effect of LPS on steady state mRNA of several representative zinc transporters (ZnT 1,4 and 6) and importers (ZIP 6,8,10,14). Using real time PCR, in two subcultures of SPAEC (one of which is shown in Fig 4A), we noted detectable levels of all of three transporters and four importers. Only ZIP14 appeared to be affected by 4 h of LPS (Fig 9A), and accordingly we expanded our studies on ZIP14 or SLC39A14. We noted that ZIP14 expression (normalized to the house keeping gene, glucose-6-phosphate dehydrogenase) was not affected by exogenous zinc (in the presence of the zinc ionophore, pyrithione), but was increased approximately 5 fold by LPS (Fig 9B). The effect of LPS was abolished by the addition of exogenous zinc (Fig 9B) suggesting that LPS induced increases in ZIP14 mRNA may be secondary to the decreases in [Zn_i] as shown above (Figures 6-8). This was supported by the sensitivity of ZIP14 mRNA to TPEN; an effect, in itself, that was reversible with exogenous zinc (Fig 9C). Indeed TPEN could augment the effect of LPS on increases in ZIP14 mRNA when administered simultaneously (data not shown).

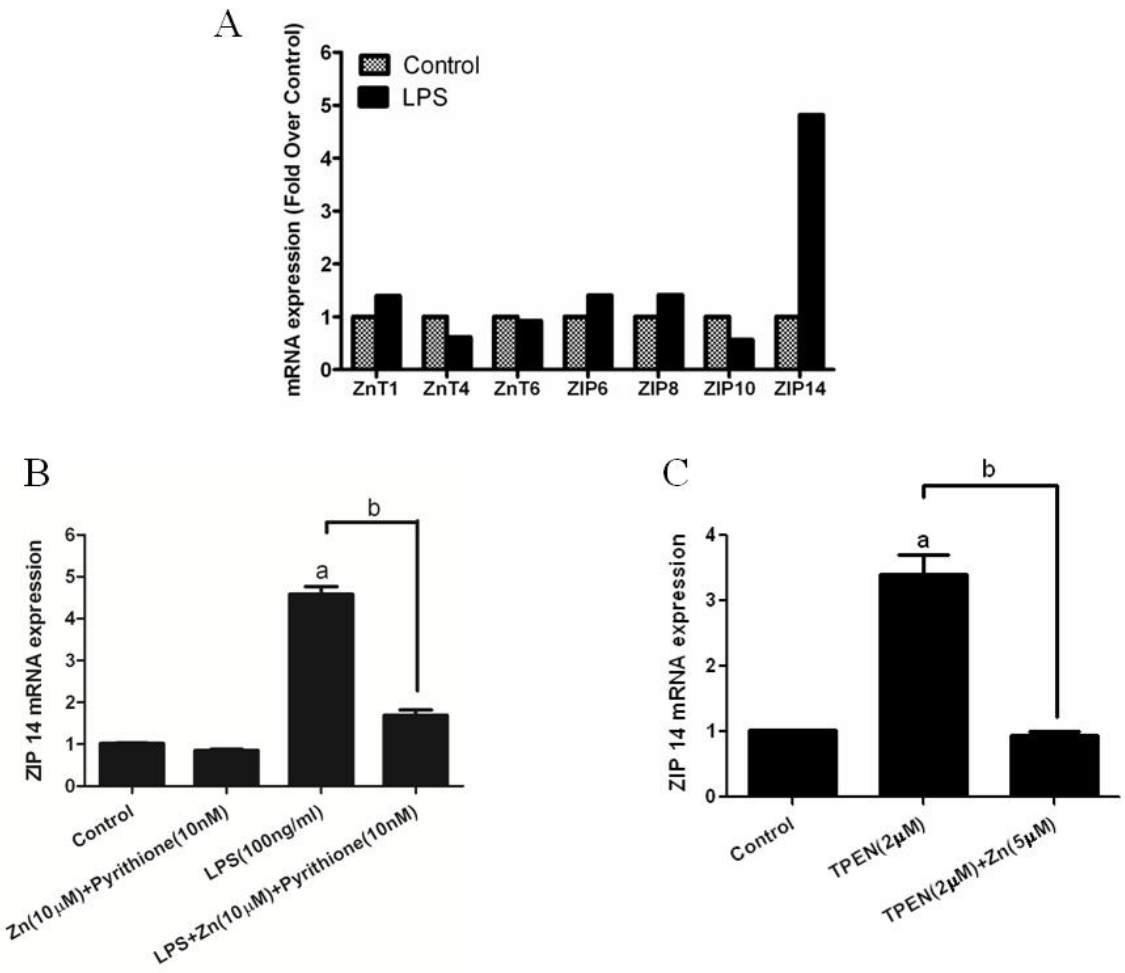

Figure 9. The effect of LPS and decreased labile [Zn_i] on representative zinc exporters and importers

Figure 9. (A) SPAECs were exposed to LPS (100 ng/ml) or HBSS (Ca^{2+}/Mg^{2+}) for 4 h. otal RNA was isolated and ZnT1,4 and 6 and ZIP 6,8,10 and 14 mRNA levels were measured by real-time PCR using specific primers and normalized to the house keeping gene G6PD. Figure 9A is representative of one of two subcultures analyzed in triplicate. (B) Effect of LPS (and its reversibility with exogenous zinc) on ZIP14 mRNA. Values are mean $\pm$ SEM of mRNA expression of samples measured in triplicate for 4 separate subcultures. (C) Effect of TPEN (and its reversibility with exogenous zinc) on ZIP14 mRNA. Values are mean $\pm$ SEM of mRNA

expression of samples measured in triplicate for 4 separate subcultures ([a] designates $p < 0.001$ compared to control; [b] designates $p < 0.001$ compared to LPS and TPEN treatment respectively; one-way ANOVA-Tukey).

3.3.5 LPS initiates apoptosis via intrinsic mitochondria-dependent pathway

We previously (70) reported that 100 ng/ml LPS caused DNA damage at 4 h in SPAEC as revealed by nuclear morphology, in situ labeling by break extension and internucleosomal DNA fragmentation. To confirm that LPS caused apoptosis in SPAEC, we used FACS analysis of annexin-V binding and showed (Fig 10A) that there was a time dependent increase in annexin-V positive (and propridium iodide negative) SPAEC over 24 h. LPS-induced annexin-V binding was sensitive to Z-VAD, a pan-caspase inhibitor, from 4-24 h, consistent with a process of apoptosis (Fig 10A). This process appeared to be intrinsic mitochondria-dependent as cytosolic cytochrome increased in a time dependent fashion after LPS (Fig 10B). Accordingly, we studied the effects of $[Zn_i]$ on early (4 h) aspects (e.g. caspase 3/7 activation, see below) of LPS-induced apoptosis in SPAEC.

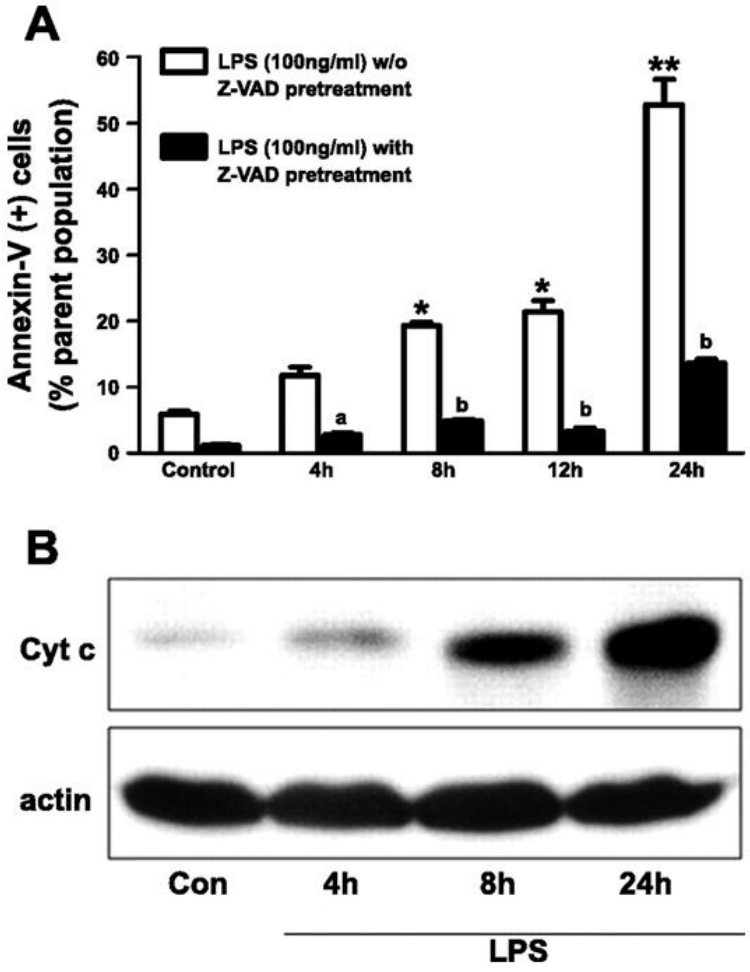

Figure 10. LPS causes intrinsic mitochondria-dependent apoptosis in SPAEC. Panel A. LPS causes a time dependent Z-VAD sensitive increase in annexin-V binding in SPAEC

Figure 10. SPAECs were treated LPS (100ng/ml) in HBSS (Ca^{2+}/Mg^{2+}) for 4, 8, 12 and 24 h. Control cells received HBSS (Ca^{2+}/Mg^{2+}) for 24 h. Apoptosis was determined by measuring PS externalization (by Annexin-V labeling) and reported as PS positive, PI negative percentage of total cell population (via FACS). Four experiments were performed on two subcultures and values are mean $\pm$ SEM. ***** and ****** designates $p < 0.01$ and $p < 0.001$ compared to control respectively; # designates $p < 0.05$ compared to control. [a] designates $p < 0.05$ and [b] designates p<

0.001 compared to without Z-VAD pretreatment (24h). Comparisons were made with two-way ANOVA-Bonferroni). Panel B. LPS mediates cyt c release in cytosol of SPAEC. One subculture of SPACE wasexposed to LPS (100ng/ml) for 4, 8 and 24 h in HBSS (Ca^{2+}/Mg^{2+}). Control cells received HBSS (Ca^{2+}/Mg^{2+}) for 24 h. Total protein was isolated and cyt c and β-actin level were determined by western blot analysis.

3.3.6 LPS-induced decreases in labile [Zn_i] contribute to apoptosis in SPAEC

As previously reported (68-70), acute treatment (4 h) with LPS did not affect cell viability as determined by Alamar Blue (Fig 11A or propidium iodide, data not shown). In figure 11B, we note that LPS caused a significant increase in caspase 3/7 activity at 4 h. Although zinc (in the presence of pyrithione), by itself, did not affect caspase 3/7 activity, it did rescue the effect of LPS on caspase 3/7 activity, consistent with our hypothesis that decreases in [Zn_i] contributed to LPS mediated apoptosis. This was further confirmed by noting that a concentration of TPEN (2 uM) sufficient to greatly reduce [Zn_i] as ascertained by FluoZin-3 (Fig 6C) was sufficient to cause comparable increases to LPS in caspase 3/7 activity; and the effect of TPEN was sensitive to exogenous zinc (Fig 11C). We (146) previously ascribed such a TPEN sensitive effect to a process of apoptosis in SPAEC.

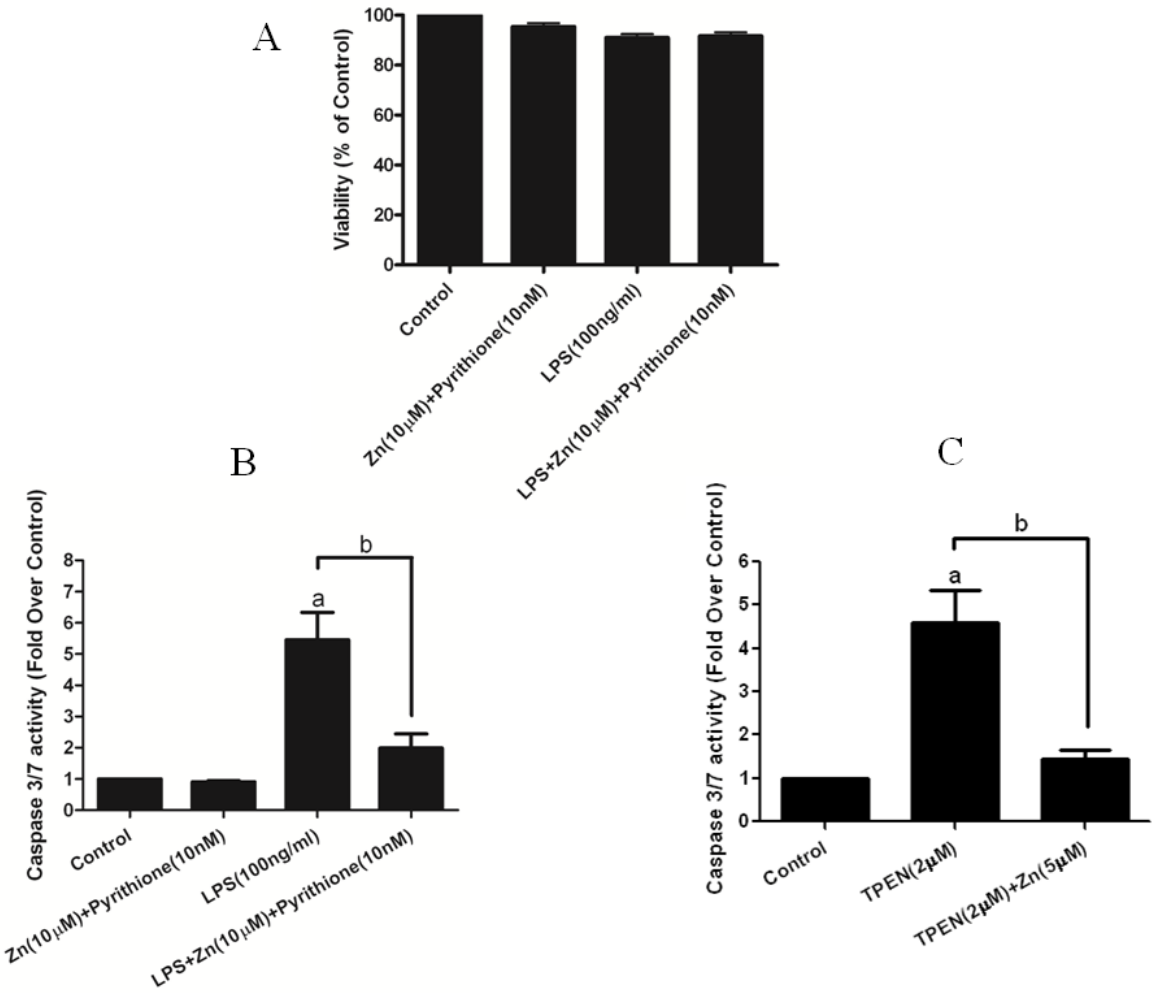

Figure 11. LPS activates Caspase3/7 in a zinc dependent fashion

Figure 11. (A) Lack of effect of LPS (or exogenous zinc) on cell viability in SPAEC. Cell viability was assessed by Alamar Blue. Data represents mean ± SEM of Alamar Blue fluorescence (% of control) of samples measured in triplicate for 4 independent experiments. (B). LPS activates caspase 3/7 in SPAEC. SPAECs were treated with LPS (100ng/ml) or HBSS (Ca^{2+}/Mg^{2+}) for 4 h and caspase 3/7 activity was measured as described. Data represents mean $\pm$ SEM of caspase-3/7activity (fold over control) of samples measured in triplicate for 4 independent experiments. (C). Zinc chelation activates caspase 3/7 activity in SPAEC. SPAEC

were treated with TPEN (2 μM) with and without 5 μM zinc added to medium for 4 h and caspase 3/7 activity was measured as described. Data represents mean ± SEM of caspase-3/7activity (fold over control) of samples measured in triplicate for 4 independent experiments ([a] designates $p < 0.001$ compared to control; [b] designates $p < 0.01$ compared to LPS treatment (panel B) or TPEN (panel C) by one-way ANOVA-Tukey.

3.4 DISCUSSION

To examine the function of zinc homeostasis in pulmonary endothelial cell apoptosis, we exposed SPAECs to LPS (100 ng/ml) for 4 h and then monitored changes in [Zn_i] by chemical (labile zinc fluorophore, FluoZin-3) and genetic approaches (zinc-sensitive chimeric reporter and ZIP14 mRNA) assays. Epifluorescence microscopy (Fig. 6) revealed that LPS causes a decrease in [Zn_i] in live cells that was significant at 2 h. This was confirmed in larger number of cells by complementary methodology of flow cytometry (Fig. 7). Additional confirmation of LPS-induced decreases in [Zn_i] was achieved by non-chemical genetic approaches (Figs 8 and 9). The functional significance of LPS induced decreases in [Zn_i] was revealed by the ability of exogenous zinc to rescue LPS-induced activation of caspase 3/7 (Fig 11B).

3.4.1 Labile [Zn_i] and signal transduction

The role of labile [Zn_i] in signal transduction is an emerging area in cell biology (59, 60, 114) and is fostered in part by: a) intricacies of zinc homeostasis including large family of importers,

transporters and binding proteins (96); b) extraordinary large number of potential regulatory targets (approximately 3-10% of genome; (2); c) association of chronic (and rare) disorders with altered zinc homeostasis secondary to mutations/variants in zinc importers and transporters and/or interactions with altered zinc nutritional status (39); and d) facileness of inorganic chemistry and coordination dynamics of zinc in affecting protein function (108). Major limitations in the field are the lack of: a) readily available fluorophores that are quantitative as well as specific for putative labile [Zn_i] (77, 79) and b) zinc-specific chemical chelators. While progress has been made in both these areas (171) many studies define labile [Zn_i] as that compartment that can alter relative fluorescence of a variety of zinc sensitive fluorophores (including FluoZin-3) and is sensitive to chelation by membrane permeant compounds such as TPEN. In this regard, advantageous features of FluoZin-3 include its selectivity (K_d=15nM) for zinc (e.g., magnesium, calcium, iron do not bind to the dye at concentrations well above what these cations may reach inside mammalian cells) (38, 179). Binding of FluoZin-3 to labile zinc is unaffected by low intracellular pH or oxidants (38, 76). Nonetheless, it is a non ratiometric dye and thus issues of loading, bleaching and lack of quantitative calibration persist. Furthermore as with all detectors of this nature, one has to introduce a potential new buffer (of zinc). As such, we took extra steps to confirm relative changes using microscopy (Fig 6) and FACS (Fig 7; to recruit large number of cells) as well as genetic (Fig 8: pLucMSC/MRE; Fig 9: mRNA ZIP14) to increase our confidence that LPS caused a decrease in [Zn_i].

3.4.2 LPS and [Zn_i]

Most studies examining a signal transduction role for labile [Zn_i] followed the paradigm of calcium homeostasis, the other major intracellular non-redox active divalent cation, and looked

for rapid and large increases secondary to extracellular flux or more often due to release from intracellular stores (172). In the case of LPS treated pulmonary endothelial cells, we initially (146) did not detect changes in labile [Zn_i] 30 min post-LPS treatment. In the current study, there were no changes up to 2 h (Fig 6). Haase et al (58) did detect increases in labile [Zn_i] 30 min post-LPS in human monocytes and granulocytes, but not lymphocytes. In this same report, they noted even earlier increases (within two minutes) in murine macrophage cell line. Nonetheless, like the original report of Kitamura et al (81) who detected decreases in labile [Zn_i] 6 hrs after treating mouse dendritic cells with LPS, we noted relative delayed decreases in labile [Zn_i] at 2-4 h in SPAEC. This delayed effect was not likely due to artifacts of FluoZin-3 as it was reproducible with MRE-Luc chimera (Fig 8) and we suggest it may be important for the signaling events associated with cell death and apoptosis. The mechanism underlying this delayed LPS-mediated decrease in labile [Zn_i] remains unclear. It is possible that an imbalance due to changes in members of ZnT or ZIP families might underlie our observations but we did not detect LPS-induced increases in mRNA in the former or decreases in the latter group, respectively. Indeed, it appears that ZIP14 mRNA increased (Fig 9A and 9B), presumably secondary to a decrease in labile zinc (as it was mimicked by TPEN, Fig 9C), suggesting a possible important feedback loop in which ZIP14 expression (perhaps via zinc sensitive promoter regions) is modulated to maintain [Zn_i] homeostasis. Current available information on regulation of ZIP14 expression (55) is limited to the role of IL-6 (96) and IL-1 (97) in hepatocytes with an obligatory contribution of NO signaling (via AP-1) affecting ZIP14 expression. We did not examine the potential of such autocrine effects to LPS in our system. It is possible that LPS affected ZnT or ZIP family members at a posttranslational level but the

limited availability of antibodies and the lack of electrophysiological assessment of zinc transport make such studies challenging.

3.4.3 Zinc and cell death

Zinc has been a primary anti-apoptotic molecule since the process of apoptosis was described (29). Subsequently earlier and more subtle zinc sensitive targets including caspase-3 (122) and poly(ADP-ribose)polymerase (144) emerged as candidates for such antiapoptotic activity of zinc. A detailed examination in HL-60 cells by Duffy et al (40) provided convincing evidence that decreases in intracellular zinc preceded early indicators of apoptosis in transformed human promyelocytic cell (HL60). We (146) and Virag et al (164) both concluded that high levels of intracellular zinc contributed to necrosis and low levels were pro-apoptotic. Nonetheless, in dendritic cells, modest (<100 uM) levels of exogenous zinc activated acid sphingomyelinase leading to production of ceramide and apoptotic cell death (133). In fetal SPAEC, hydrogen peroxide induced increases in zinc were associated with apoptosis and this effect was blunted by zinc chelators including TPEN or overexpression of metallothionein (169). This suggests that some effects of zinc and apoptosis are cell specific and/or dependent upon developmental stage. As has been shown in systemic endothelium using different pro-apoptotic stimuli (111), in LPS treated SPAEC, TPEN exacerbates apoptosis (146) and exogenous zinc can rescue this phenotype (Figure 11B). In the more complex scenario in which nitric oxide is introduced into the milieu of LPS treated SPAEC, we (147) noted that NO mediated resistance to LPS was Zn dependent suggesting that elevations in labile [Zn_i] secondary to either influx from extracellular

sources or chemically mediated release of zinc perhaps from metallothionein (138) can produce LPS resistant phenotype.

3.4.4 Zinc and acute lung injury

Zinc deficiencies increase the susceptibility of experimental animals to hyperoxic (148), alcohol (74) and sepsis (82) -induced lung injury. We (93) and others (177) have reviewed the role of zinc in acute lung injury and the majority of our insight is related to airway epithelium (156, 157). The proximity of a labile pool of zinc and pro-caspase-3 in ciliary basal bodies of airway epithelium and the ability of TPEN to cause apoptosis or exacerbate other pro-apoptotic stimuli provided support for an anti-apoptotic role of labile zinc (19, 48, 159) in airway epithelium. Further details of an anti-apoptotic role for zinc in human airway epithelium were provided by Bao and Knoell (5, 6) who reported zinc depletion exacerbated apoptosis and decreased barrier function secondary to Fas antibody, TNFα and IFNγ. These authors extended these studies to show that zinc depletion augmented acute lung injury (including apoptosis, enhanced inflammation, altered innate immunity) in polymicrobrial sepsis in mice (7, 82). Collectively, these reports underscore the importance of zinc metabolism in the airway and lung and suggest that zinc supplementation may be of therapeutic utility and further insights into zinc homeostasis may reveal critical aspects of pathogenesis in acute and chronic lung disorders (174).

3.5 CONCLUSION

Our findings show for the first time that a decrease in labile zinc in pulmonary endothelium is an important signaling event in LPS-induced apoptosis. Along with the report of Kitamura et al (81), it suggests that such a change in labile [Zn_i] may be an important mechanism by which cells respond to exogenous stimuli such as LPS. Critical future efforts should be directed towards understanding the mechanism by which [Zn_i] decreases, the cellular targets affected by such a decrease and the overall physiological processes (in addition to apoptosis) that occur in the setting of LPS and pulmonary endothelium.

3.6 ACKNOWLEDGEMENTS

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4.0 A CRITICAL ROLE FOR INCREASED LABILE ZINC IN REDUCING SENSITIVITY OF CULTURED SHEEP PULMONARY ARTERY ENDOTHELIAL CELLS TO LPS-INDUCED APOPTOSIS.

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4.1 ABSTRACT

We previously noted an important signaling role for decreased labile intracellular zinc ($[Zn]_i$) in LPS-induced apoptosis in cultured sheep pulmonary artery endothelial cells (SPAEC; (147, 153)). In the current study, we used siRNA to important contributors of zinc homeostasis (SLC39A14 or ZIP14, a zinc importer; metallothionein (MT), a zinc binding protein) to identify molecular pathways by which extracellular zinc or nitric oxide (NO) increase labile $[Zn]_i$ (e.g. zinc-sensitive fluorophore (FluoZin-3) detectable and/or chelatable by N,N.N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN)) and reduce the sensitivity of SPAEC to LPS. Addition of 10 μM zinc to serum free medium of SPAEC increased $[Zn]_i$ and abolished LPS-induced apoptosis (e.g., increased annexin-V binding). The increase in $[Zn]_i$ and the protective effect of extracellular zinc were sensitive to reduction in ZIP14 expression (by siRNA) but not affected by collectively knocking down major isoforms of sheep MT (sMT-Ia,-Ib,-Ic,and -II). Pretreatment of wildtype SPAEC with 250 μM of the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) increased labile zinc in a relatively similar fashion to addition of extracellular zinc and reduced sensitivity of SPAEC to LPS-induced apoptosis (e.g. caspase-3/7 activation) in a TPEN sensitive fashion. The antiapoptotic effects of SNAP were insensitive to siRNA knockdown of ZIP14 but were abolished (along with SNAP-induced increase in $[Zn]_i$) when SPAEC were pretreated with siRNA to sheep MT. Zinc was able to directly inhibit recombinant caspase-3 activity in an in-vitro assay. Collectively, these data show that increases in labile $[Zn]_i$ are an important component of ZIP14- or NO-mediated resistance to LPS-induced apoptosis. Cytoprotection via ZIP14 appeared to be secondary to transcellular movement of extracellular zinc whereas NO-mediated protection was secondary to S-nitrosation of MT and redistribution of intracellular zinc.

4.2 INTRODUCTION

Zinc is an essential micronutrient whose total intracellular levels are maintained in a tight quota from 100 to 500 μM (118). Although zinc-binding proteins have been reported to be part of 10% of the entire proteome (2), the major determinants (51) of the critical, dynamic and vanishingly small (and hence the descriptor, "trace") labile pool of intracellular zinc ($[Zn]_i$) appear to be a family of zinc importers (SLC39A1-14; Zrt/Irt-like protein or ZIP), transporters (SLC30A1-10; ZnT) and metal binding protein, metallothionein (MT). The labile or transient pool (operationally defined as the compartment chelated by molecules such as N,N.N',N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) and/or detected by nonprotein-bound zinc-sensitive fluorophores such as FluoZin-3 (153)) is thought to function as a signaling molecule (in an analogous fashion to better studied metals such as calcium (66)) and contributes to diverse processes including neurotransmission, insulin secretion, fertilization and immune function (51). We have reviewed the role of zinc homeostasis in acute lung injury (93, 136) and have particularly focused on the role of $[Zn]_i$ in pulmonary endothelial cell function including contraction (12) and cell death (146, 147, 153).

We have used a simple model of primary cultures of sheep pulmonary artery endothelial cells (SPAEC) to show that: a) extraordinarily high levels of $[Zn]_i$ (after addition of zinc to medium) cause cell death by necrosis (146); b) very low levels of $[Zn]_i$ (after TPEN) cause apoptosis (146); and c) LPS-induced apoptosis (68-70) is associated with a critical time dependent decrease in intracellular $[Zn]_i$ (153). We have been able to inhibit LPS-mediated apoptosis by exposing SPAEC to supplemental zinc (along with zinc ionophore, pyrithione) in the medium (153), exposing SPAEC to nitric oxide donors (147) or after gene transfer of

inducible nitric oxide synthase (20, 160). Collectively the protective effects of addition of extracellular zinc or conditioning with NO have in common potential increases in $[Zn]_i$ (138, 146) but the mechanism by which $[Zn]_i$ increased in a manner sufficient to inhibit LPS-induced apoptosis remained unclear.

In the current study, we reveal singularly important and discrete contributions of SLC39A14 (ZIP14) and MT, in affecting the ability of extracellular zinc or nitric oxide, respectively, to inhibit LPS-induced apoptosis in SPAEC. By silencing ZIP14 or sheep MT isoforms, we revealed not only the manner, respectively, by which extracellular zinc or nitric oxide produce an elevation in $[Zn]_i$ but also the central role of zinc dyshomeostasis in LPS-induced apoptosis in SPAEC.

4.3 RESULTS

4.3.1 Extracellular zinc inhibits LPS-induced apoptosis in SPAEC in a ZIP14 (but MT independent) dependent fashion.

We (153) previously reported that addition of 10 µM zinc (with 10 nM pyrithione) to the medium of SPAEC was sufficient to prevent apoptosis at 4 h after exposure to LPS. We now show that 10 µM zinc (in serum free medium without pyrithione) increases labile $[Zn]_i$ (Figure 12) and protects SPAEC for 24 h from the progressive (153) increase in LPS-induced apoptosis as reflected in annexin-V positive cells (Figure 13). Of note is that exogenous zinc significantly inhibited spontaneous apoptosis due to experimental conditions (i.e. prolonged culture of SPAEC in serum free medium).

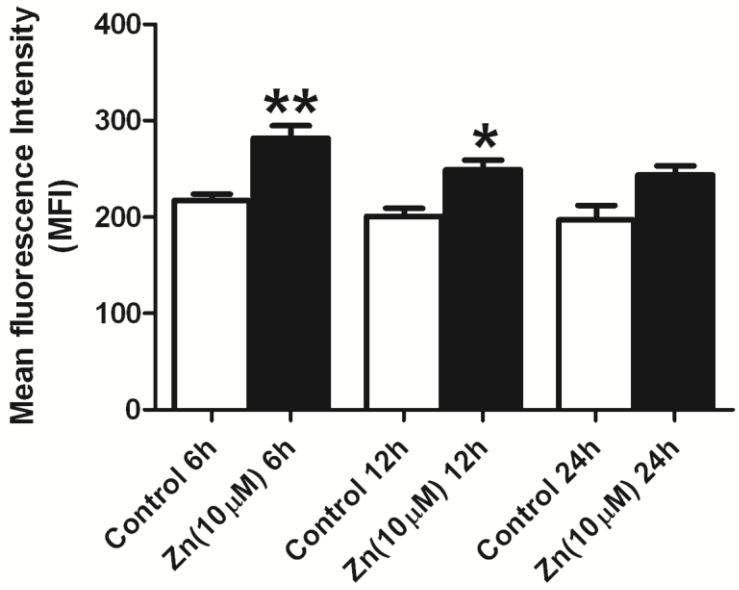


Figure 12. Fluorescence of FluoZin-3 in response to 10 μM zinc in a time dependent fashion

Figure 12. SPAEC was treated in the presence or absence of zinc (10 μM) for 6 h, 12 h and 24 h respectively. Following treatment, cells were incubated with FluoZin-3 AM and equal volume of Pluronic F-127 (20 minutes; 37 °C). Cells were trypsinized, centrifuged and injected into FACS. Extracellular zinc (10 μM) increased fluorescence of FluoZin-3 by 29.6% (6 h), 24.1% (12 h) and 23.6% (24 h). Data represents mean fluorescence intensity (MFI) of FluoZin-3 in up to 20,000 cells from 6-7 independent experiments for each time point. **$p < 0.001$ and *$p < 0.01$ compared to time point controls; one-way ANOVA-Tukey.

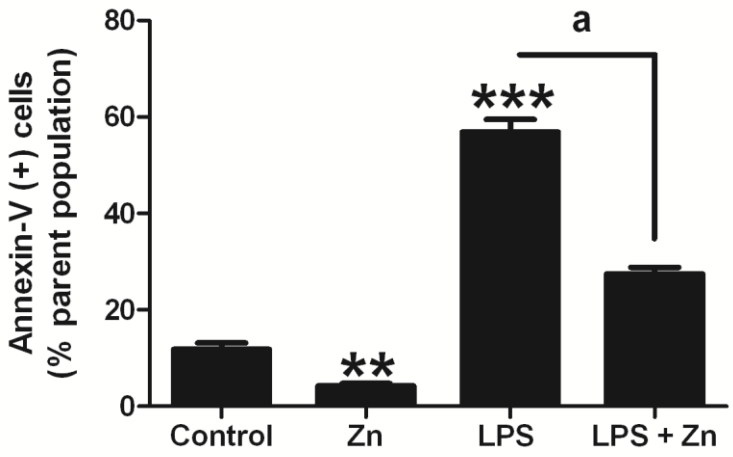


Figure 13 . LPS- (100 ng/ml; 24 h) induced apoptosis as determined by Annexin-V assay

Figure 13. SPAEC was treated with LPS (100 ng/ml; 24 h) in the presence or absence of zinc (10μM; 24h) or zinc alone (10 μM; 24 h). Following treatments, cells were trypsinized, centrifuged and resuspended with buffer containing Annexin-V-FITC and propidium iodide (PI) and analyzed by FACS. Data represent Annexin-V positive cells (mean+SE) from 8 independent experiments. **p< 0.001 and ***p< 0.0001 compared to control; [a] p< 0.0001 compared to LPS treatment; one-way ANOVA-Tukey.

In our previous report (153), we noted that ZIP14 increased at mRNA level in response to LPS induced decrease in labile $[Zn]_i$ suggesting a possible homeostatic role for ZIP14. Accordingly, we designed siRNA against ZIP14 (using bovine genomic information) that significantly decreased ZIP14 mRNA to 40% of baseline levels (Figure 14) in SPAEC. siRNA to ZIP 14 significantly decreased basal levels of labile $[Zn]_i$ and blocked zinc uptake (relative to scramble siRNA group) in SPAEC treated with 10 μM zinc (Figure 15). In figure 16, we show that zinc dependent inhibition of spontaneous and LPS-induced apoptosis (at 24 h) was abolished after SPAEC were treated with siRNA to ZIP14. In contrast, SPAEC treated with scrambled siRNA were still responsive to zinc inhibition of spontaneous and LPS-induced apoptosis (Figure 16) in a manner similar to wildtype SPAEC (Figure 13).

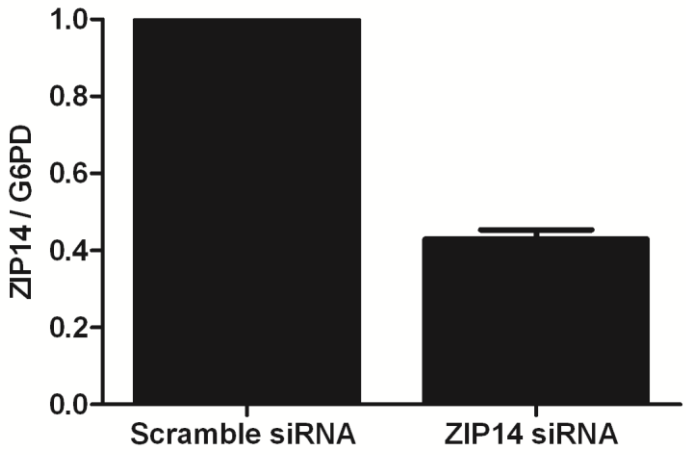


Figure 14. ZIP14 mRNA levels in ZIP14 siRNA treated SPAECs

Figure 14. SPAECs were transfected with lipofectamine RNAimax plus scramble or ZIP14 siRNA (40nM). 72 h after transfection total RNA was isolated and ZIP14 mRNA levels were measured by qPCR using specific primers and normalized to the house keeping gene G6PD.

Data represents mRNA expression (mean + SEM) of samples measured in triplicates from 5 independent experiments.

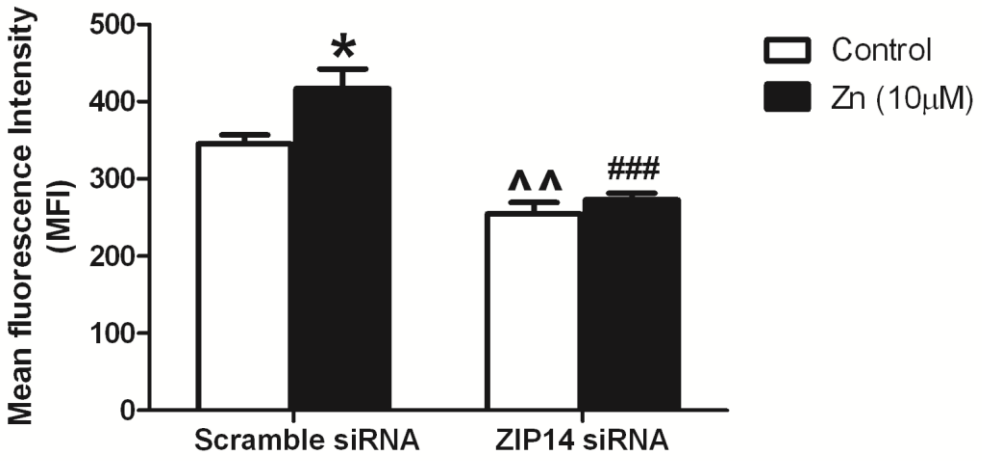


Figure 15. Fluorescence of FluoZin-3 in response to zinc (10 μM; 6 h) in cells treated with siRNA to ZIP14

Figure 15. SPAEC was treated with or without zinc (10 μM) for 6 h. Following treatment, cells were incubated with FluoZin-3 AM and equal volume of Pluronic F-127 (20 minutes; 37 °C). Cells were trypsinized, centrifuged and injected into FACS. Extracellular zinc (10 μM) increased fluorescence of FluoZin-3 by 21% (6 h) in scramble siRNA treated cells. Data represents mean fluorescence intensity (MFI) of FluoZin-3 in up to 20,000 cells from 6 independent experiments. *p< 0.05 compared to scramble siRNA control; ^^p<0.001 compared to scramble siRNA control; ###p<0.0001 compared to scramble siRNA zinc treatment; two-way ANOVA-Bonferroni.

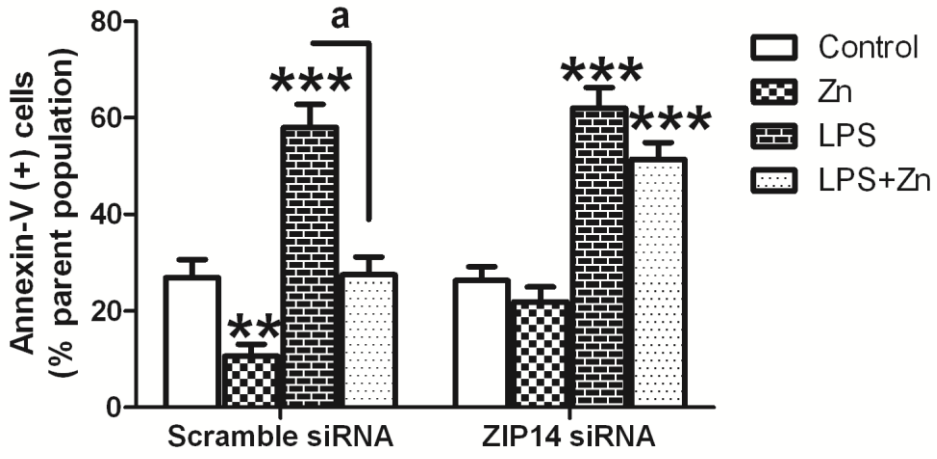


Figure 16. LPS- (100 ng/ml; 24 h) induced apoptosis in cells treated with siRNA to ZIP14 as determined by Annexin-V assay

Figure 16. SPAEC was transfected with scramble siRNA (40 nM) or ZIP14 siRNA (40 nM) and then treated with LPS (100 ng/ml; 24 h) in the presence or absence of zinc (10 μM; 24 h) or zinc alone (10 μM; 24 h). Following treatments, cells were trypsinized, centrifuged and resuspended with buffer containing Annexin-V-FITC and propidium iodide (PI) and analyzed by FACS. Data represent Annexin-V positive cells (mean+SE) from 7 independent experiments. ***$p < 0.0001$ compared to control; [a] $p < 0.0001$ compared to LPS treatment; two-way ANOVA-Bonferroni.

Metallothionein is thought to play a critical role in zinc homeostasis acting as a dynamic intracellular zinc buffer (85-87, 105). Accordingly, we designed siRNA to sheep MT-Ia, -Ib, -Ic and II and were able to reduce expression of mRNA of MT-Ia,c and II to less than 10% of baseline levels (Figure 17), respectively (there was no detectable sheep MT-Ib at baseline and the fifth ovine MT gene is a pseudo gene). Under conditions of these experiments, LPS-induced apoptosis was significantly greater in MT knockdown vs scrambled siRNA treated SPAEC

(Figure 18). Nonetheless, addition of extracellular zinc was still able to rescue MT knockdown and scramble siRNA treated SPAEC (Figure 18) suggesting MT was not critical for the protective effects of altered $[Zn]_i$ after exposure to 10 µM zinc in medium.

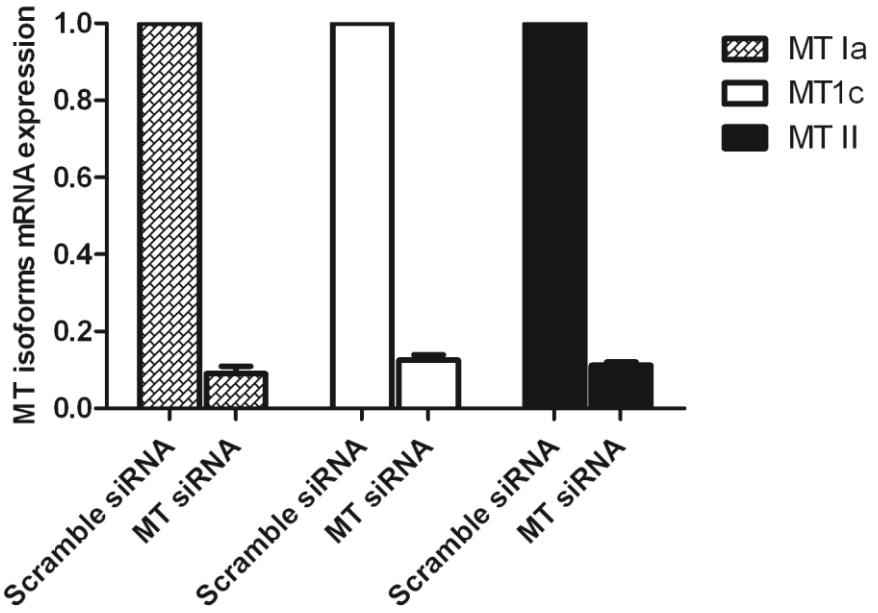


Figure 17. MT mRNA levels in MT-Ia, -Ib, -Ic and II siRNA treated SPAECs

Figure 17. SPAECs were transfected with MT- Ia,-Ib,-Ic (10nM) & MT II siRNA (10nM) or scramble siRNA (20nM). 72 h after transfection, total RNA was isolated and MT Ia,Ib,Ic & II mRNA levels were measured by real-time PCR using specific primers for each isoforms and normalized to the house keeping gene G6PD. Data represents relative mRNA expression (mean+SEM) of MT- Ia,-Ic & II in samples measured in triplicates from 3 independent experiments.

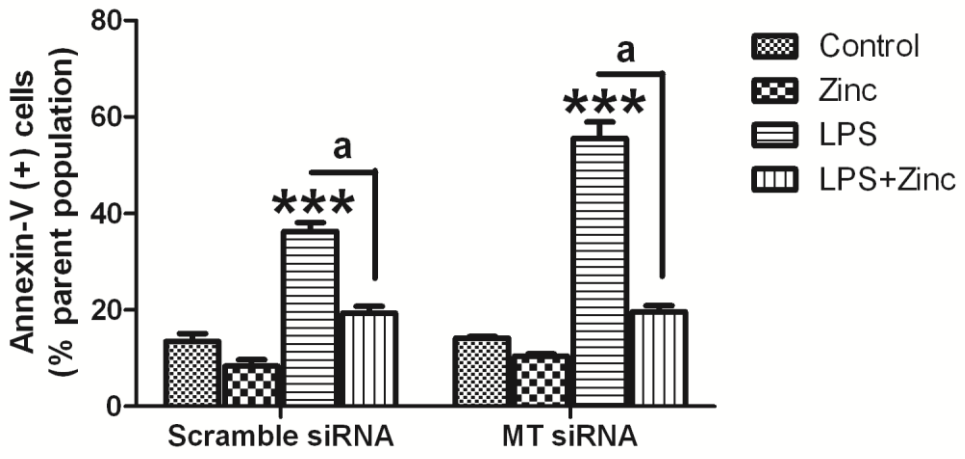


Figure 18. LPS- (100 ng/ml; 24 h) induced apoptosis in cells treated with siRNA to MT I/II as determined by Annexin-V assay

Figure 18. SPAEC was transfected with scramble siRNA (20 nM) or MT I/II (20 nM) siRNA and then treated with LPS (100 ng/ml; 24 h) in the presence or absence of zinc (10 μM; 24 h) or zinc alone (10 μM; 24 h). Following treatments, cells were trypsinized, centrifuged and resuspended with buffer containing Annexin-V-FITC and propidium iodide (PI) and analyzed by FACS. Data represent Annexin-V positive cells (mean+SE) from 3 independent experiments. ***$p < 0.0001$ compared to control; [a] $p < 0.0001$ compared to LPS treatment; two-way ANOVA-Bonferroni.

4.3.2 Nitric oxide inhibits LPS-induced apoptosis in SPAEC in an MT- and Zn^{2+}-dependent (but ZIP14 independent) fashion

We (147) previously noted that treatment with SNAP (500 μM), a slow NO releaser ($t_{1/2}$ ~ 6 h), reduced sensitivity of wildtype SPAEC to LPS-induced apoptosis (as determined by nuclear morphology). Reversal with large (10 μM) doses of TPEN were consistent with a potential role of zinc in mediating such resistance. In the current study, we extend these observations and

show that lower (250 µM) doses of SNAP pretreatment were protective against relatively high (1 µg/ml) doses of LPS-induced apoptosis at 4h (Figure 19) as well as lower (100 ng/ml) doses of LPS at 24h (Figures 20a-b). In this latter instance, SNAP was equally effective whether SPAEC were incubated in serum (Figure 20a) containing approximately 3-11 µM zinc (26, 53) or in zinc free HBSS (Figure 20b). Furthermore, considerably lower and non-toxic doses (1 µM) of TPEN abrogated SNAP-induced resistance (Figure 19) in a fashion equally effective to potentially toxic (146) dose (10 µM) of TPEN. Similar results were obtained if annexin-V binding (via FACS) instead of caspase 3/7 activation was used to quantify apoptosis (data not shown).

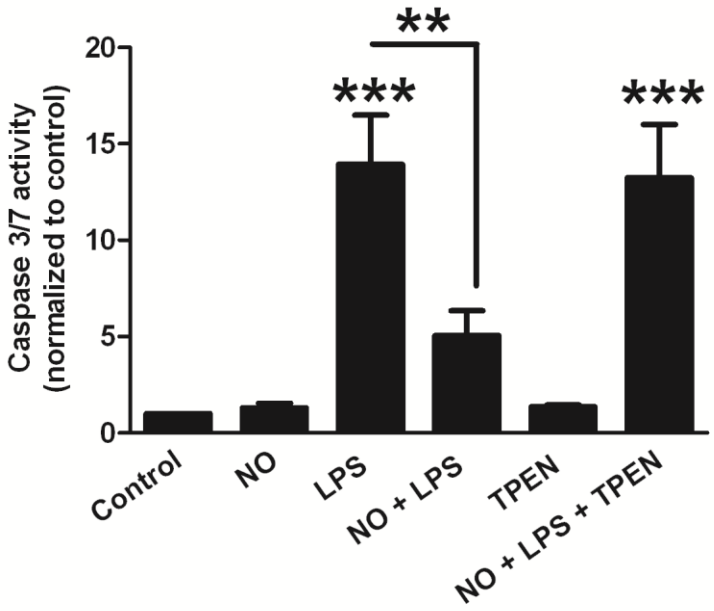


Figure 19. NO (250 µM; 6 h) -mediated protection against LPS (1 ug/ml; 4 h) as determined by caspase 3/7 assay

Figure 19. SPAEC was treated with LPS (1 µg/ml; 4 h). The protective effect of NO on LPS-induced apoptosis was assessed by pretreatment with NO donor, SNAP (250 µM; 6 h). The NO mediated protection was reversed by nontoxic dose of zinc chelator, TPEN (1 µM; 4 h). Apoptosis was determined by measuring caspase-3/7 activity. Data represents caspase-3/7

activity (mean+SE) of samples for 5 independent experiments. ***p < 0.0001 compared to control; ** p< 0.001 compared to LPS treatment; one-way ANOVA-Tukey.

Figure 20A

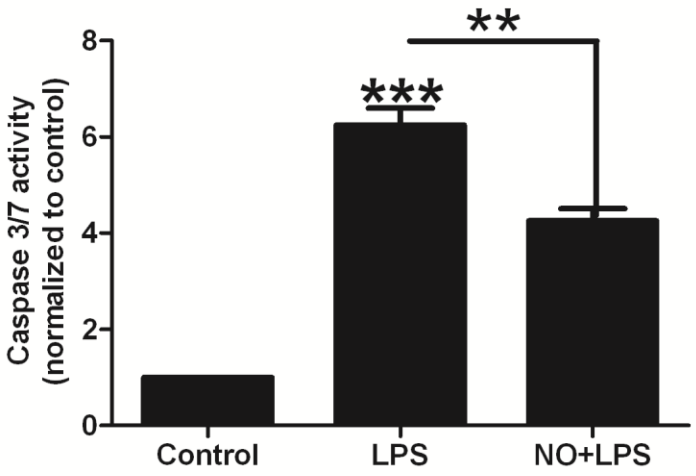


Figure 20B

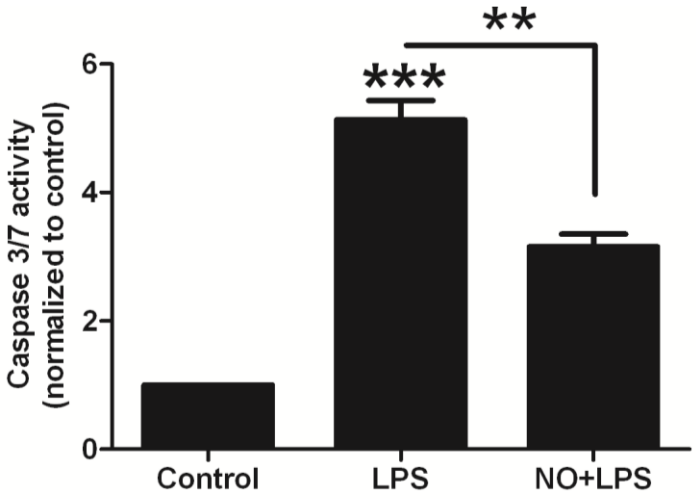


Figure 20. NO (250 μM;6 h) -mediated protection against LPS (100 ng/ml; 24 h) as determined by caspase 3/7 assay

Figure 20. SPAEC was pretreated with NO donor, SNAP (250 μM; 6 h) in serum (A) or in HBSS (B) prior to LPS (100 ng/ml; 24 h). Apoptosis was determined by measuring caspase-3/7

activity. Data represents caspase-3/7 activity normalized to control (mean+SE) of samples for 3 independent experiments. ***p < 0.0001 compared to control; ** p< 0.001 compared to LPS treatment; one-way ANOVA-Tukey

In figure 21, we note that NO-mediated protection against LPS at 4 h (as determined by annexin-V binding) was not significantly affected by silencing of ZIP14. Although NO has been reported by others (97) to increase expression of ZIP14 in hepatocytes, there were no significant changes in ZIP14 mRNA at 6 h (as measured by RT-PCR and normalized to G6PD) after 250 to 1000 µM SNAP (Figure 22).

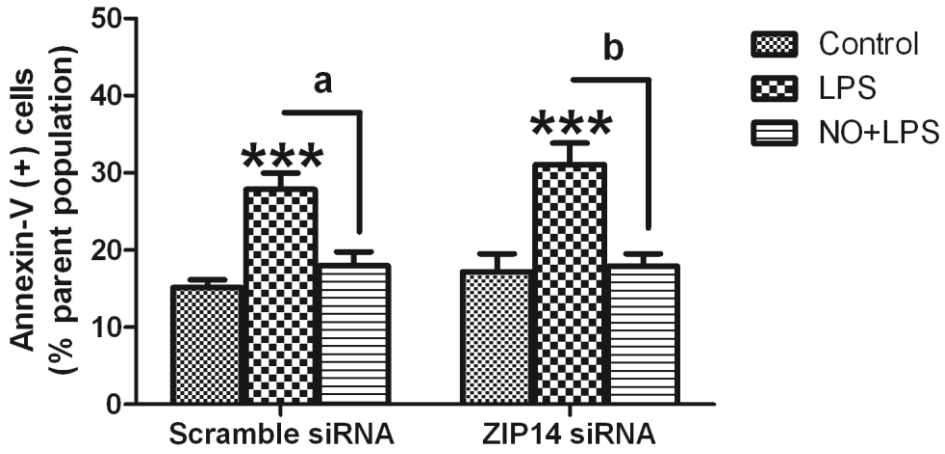


Figure 21. NO-(250 µM;6 h)mediated protection against LPS (1 ug/ml; 4 h) is independent of ZIP14 as determined by Annexin-V assay

Figure 21. SPAEC was transfected with scramble siRNA (40 nM) or ZIP14 siRNA (40 nM) and then exposed to LPS (1 ug/ml) with or without NO donor, SNAP (250 µM; 6 h) pretreatment. Following LPS exposure, cells were trypsinized, centrifuged and resuspended with buffer

containing Annexin-V-FITC and propidium iodide (PI) and analyzed by FACS. Data represent Annexin-V positive cells (mean+SE) from 11-13 independent experiments. ***p < 0.0001 compared to control; [a] p <0.001 and [b] p< 0.0001 compared to LPS; two-way ANOVA-Bonferroni.

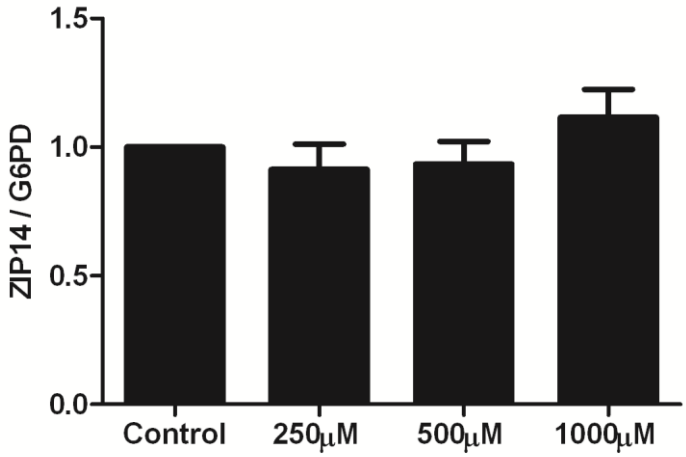


Figure 22. ZIP14mRNA in response to NO (250 μM; 6 h)

Figure 22. SPAEC was exposed to NO donor, SNAP (250 μM - 1 mM; 6 h). Total RNA was isolated and ZIP14 mRNA levels were measured by qPCR using specific primers and normalized to the house keeping gene G6PD. Data represents mean ± SEM of mRNA expression of samples measured in triplicates for 5 independent experiments.

Accordingly, we focused on a potential role for MT in affecting NO-mediated resistance to LPS-induced apoptosis since we previously reported that MT was obligatory for NO-mediated increases in $[Zn]_i$ in cultured lung fibroblasts (138) and mouse lung endothelial cells (147)

isolated from MT null mice. In figure 23, we show typical fluorescent microscopic images of SPAEC that were incubated with the zinc sensitive fluorophore, FluoZin-3, and exposed to large (2 mM) doses of SNAP for brief (10 min) periods of time. Significant increases (4-6X) in fluorescence were noted in wildtype or scramble siRNA treated SPAEC whereas notably less (<2X) change in fluorescence was noted in MT knockdown SPAEC. Relative changes in labile zinc were assessed by FACS (using Fluozin-3) after SPAEC were exposed to cytoprotective doses of SNAP (250 µM; 6 h) and a 24 +/- 5% increase ($P<0.05$), comparable to that noted after 10 µM zinc was added to the medium (Figures 12 and 14), was observed.

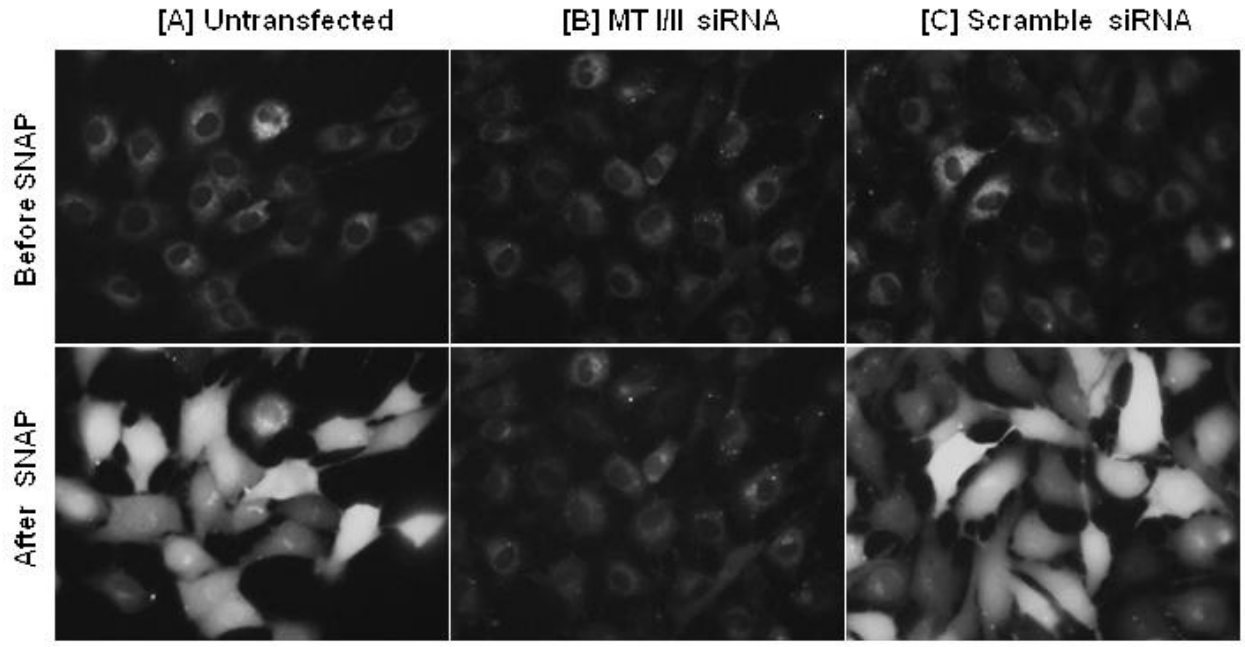
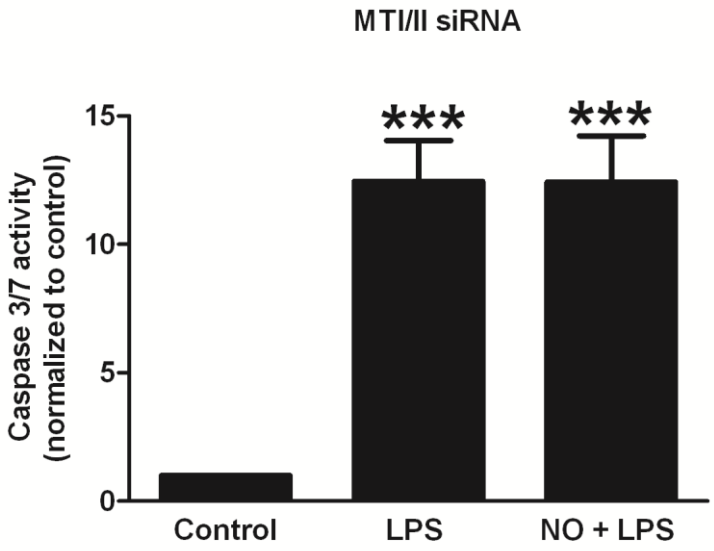


Figure 23. NO liberates zinc from MT

Figure 23. SPAEC was transfected with scramble siRNA (20 nM) or MT I/II (20 nM) siRNA. Following siRNA treatment, cells (untransfected or transfected) were incubated with FluoZin-3 AM and equal volume of Pluronic F-127 (20 minutes; 37 °C). Cells were imaged by

epifluorescence microscope before and after exposure to NO donor, SNAP (2 mM; 10 minutes). The images represent fluorescence intensity of FluoZin-3-Zn^{2+}complex in SPAECs. All images were captured with identical gain, 100% light intensity, 1 millisecond light exposure and 4x4 binning. For statistical significance increase in FluoZin-3 fluorescence were quantified in a larger number of cells (800-1000 cells) from 5 independent experiments.

LPS, by itself, was more toxic (as determined by increased caspase-3/7 activity responses; Figure 24a) in MT knockdown cells than SPAEC treated with scrambled siRNA (Figure 24b). This was in accord with observations at 24 h and using annexin-V as the determinant of apoptosis (Figure 18). SNAP was able to significantly inhibit LPS-induced apoptosis in scramble siRNA treated SPAEC (Figure 24b) but not in MT knockdown SPAEC (Figure 24a).



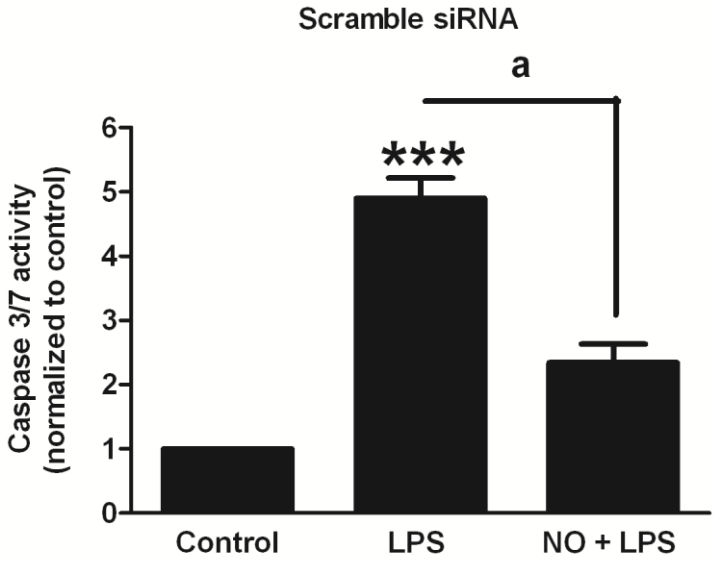


Figure 24. NO-(250 µM;6 h)mediated protection against LPS (1 ug/ml; 4 h) is dependent on MT I/II as determined by caspase 3/7 assay

Figure 24. SPAEC was transfected with (A) MT I/II siRNA (20 nM) or (B) scramble siRNA (20 nM). 72 h after transfection, cells were treated with LPS (1 µg/ml; 4 h) with or without NO donor, SNAP pre-treatment (250 µM; 6 h). The anti-apoptotic effect SNAP was abrogated in MT I/II knockdown SPAECs (Figure 24a). The NO-mediated protection was preserved in the scramble siRNA treated SPAECs (Figure 24b). Apoptosis was determined by measuring caspase-3/7 activity. Data represents caspase 3/7 activity (mean+SE) of samples from 9 independent experiments. *** designates $p < 0.0001$ compared to control; [a] $p < 0.0001$ compared to LPS; one-way ANOVA-Tukey).

4.3.3 Zinc inhibits caspase 3

To determine whether zinc had a direct inhibitory effect on caspase 3, we studied the effect of zinc on purified recombinant caspase-3. Caspase-3 was incubated with the peptide substrate DEVD.AFC, in the presence or absence of zinc. After incubation (30 minutes; RT), fluorescence of cleaved AFC was measured. As shown in figure 25, zinc inhibits the enzymatic activity of caspase-3 in a dose dependent manner such that 50% inhibition in casapse-3 activity was achieved with 200 nM zinc and such inhibition was sensitive to zinc chelator, TPEN (200 μM; 30 minutes).

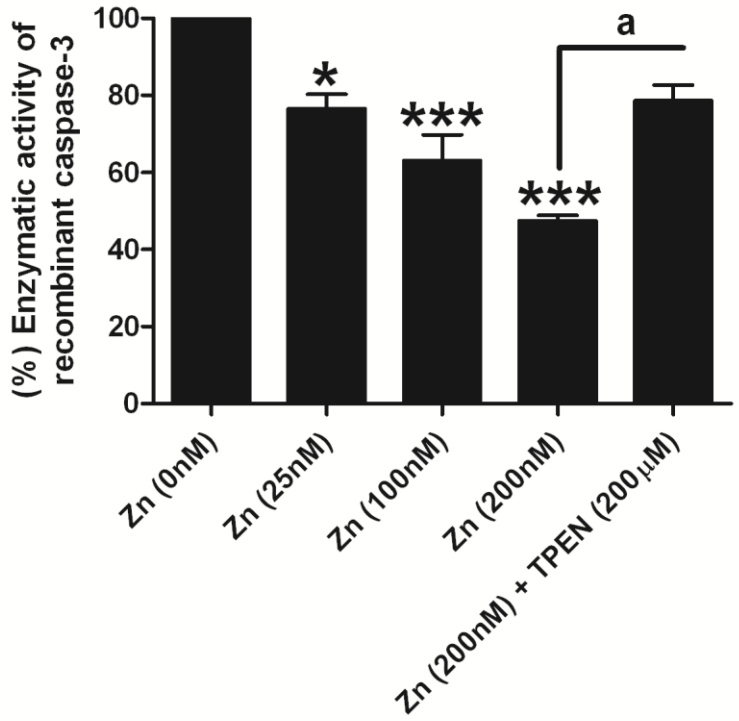


Figure 25. Zinc directly modulates caspase 3/7 activity

Figure 25. Inhibition of caspase-3 activity by zinc was studied in an in-vitro system. Purified caspase-3 enzyme was added to a reaction buffer containing DEVD.AFC in the presence or absence of the indicated concentrations of zinc. The fluorescence of the liberated AMC was monitored to determine the effect of zinc on the enzymatic activity of purified capsase-3. Data represents mean+SE of enzymatic activity of capsase-3 (expressed as % of control). (*$p< 0.05$ and ***$p<0.0001$ compared to without zinc; [a] $p<0.001$ compared to zinc (200nM); one-way ANOVA-Tukey)

4.4 DISCUSSION

We (153) recently reported that a decrease in labile $[Zn]_i$ was a critical signaling event in LPS-mediated apoptosis in SPAEC. An important observation within that study was the ability of exogenous zinc to inhibit LPS-induced apoptosis over 4h and we now extend this to a 24h period after LPS (Figure 13). Previously we (20, 160) had shown that NO can inhibit LPS-mediated apoptosis in SPAEC and such inhibition was itself reversed with TPEN (147) lending further support to an important role of labile zinc in this simple model of intrinsic apoptosis (68-70, 153). In the current study, we show for the first time that genetic reduction of MT and its resultant inhibition of NO-mediated zinc release (Figure 23) is coupled to abrogation of the ability of NO to prevent LPS- mediated apoptosis in SPAEC (Figure 24a). In combination with the requirement for full expression of ZIP14, a plasma membrane bound zinc importer, to mediate the protective effects of extracellular zinc (Figure 16), it is apparent that elevations in $[Zn]_i$, whether secondary to zinc release from intracellular stores (e.g. MT) or after translocation

of zinc across plasma membrane (via ZIP14), are important components of signaling pathways accounting for inhibition of LPS-induced apoptosis. Reduction of MT (<10% of basal levels) by siRNA in SPAEC completely inhibited the ability of NO to protect SPAEC against LPS-induced apoptosis. NO-mediated resistance was independent of ZIP14 (Figure 21) suggesting that the increase in labile zinc occurred by redistribution of cellular sources of zinc and specifically MT as outlined above. The mechanism by which elevations in labile $[Zn]_i$ are antiapoptotic remain unclear although we confirmed (122, 167) that zinc is capable of inhibiting caspase-3 in an in vitro assay (Figure 25). A schema summarizing our findings including speculative details regarding chemistry of S-nitrosation of MT and caspase-3 inhibition is provided in figure 26.

4.4.1 Extracellular zinc, ZIP14, MT and zinc homeostasis in SPAEC

$[Zn]_i$ is tightly controlled by a large family of zinc importers (SLC39A1–14 or ZIP1–14) and exporters (SLC30A1–10 or ZnT1–10) and an extraordinary large number of proteins that bind zinc. In the current study we focused on ZIP14 and metallothionein because: a) ZIP14, itself, is regulated by LPS (and attendant cytokines) in pulmonary endothelium (153) and hepatocytes (101); and b) metallothionein is considered to be a major intracellular zinc buffer by binding 7 mol of Zn^{2+}/mol of MT in a dynamic fashion (57, 105, 107). In particular, we recently reported that LPS- (100ng/ml; 4h) induced decreases in labile $[Zn]_i$ and upregulation in ZIP14 mRNA were potentially linked in SPAECs (153). LPS-induced increases in ZIP14 mRNA and apoptosis were normalized when labile $[Zn]_i$ was elevated by addition of zinc (in the presence of the zinc ionophore, pyrithione) suggesting that LPS-induced increases in ZIP14 mRNA may be secondary to the decreases in $[Zn]_i$ (as it was mimicked by TPEN). Accordingly, we expanded our study on ZIP14 in the current work and elucidated its role in LPS-induced apoptosis. Cells

exposed to LPS (100 ng/ml; 24 h) in the presence of exogenous zinc (10 μM; sufficient to increase FluoZin-3 detectable labile zinc by 20-30% over 24 h (Figure 12)) had a significant decrease in apoptosis as determined by Annexin-V labeling (Figure 13) in a ZIP14 dependent fashion (Figure 16). siRNA to ZIP14 decreased labile $[Zn]_i$ at control levels and blocked increases in labile $[Zn]_i$ in SPAEC treated with extracellular zinc (Figure 15). Indeed, under conditions of current experiments (prolonged exposure in serum-free zinc free medium after treatment with lipid mediated vectors with siRNA), spontaneous apoptosis was relatively high in the scrambled siRNA group (Figure 16) and was inhibited by exogenous zinc, itself, in a ZIP14 dependent fashion. In contrast, genetic reduction of MT expression did not affect the ability of exogenous zinc to prevent LPS-mediated apoptosis in SPAEC (Figure 18) suggesting that delivery of zinc via ZIP14 elevates cytosolic zinc in a fashion unaffected by Zn^{2+}-MT interactions (Figure 26). siRNA mediated reduction of MT expression did sensitize SPAEC to the apoptotic effects of LPS, per se (Figures 18 and 24). This latter observation is similar to the protective role of MT in acute lung injury induced by bacterial endotoxin (145). We did not study the role of other zinc importers noted to be important in inflammation including ZIP6 in human dendritic cells (81) and ZIP8 in human airway epithelial cells (13) in part because their expression at mRNA was unaffected by LPS in SPAEC (153). In addition to importing zinc, ZIP14 is a functional divalent metal ion transporter affecting non-transferrin bound iron (101, 125, 180). Our treatment medium did not contain iron as well as zinc (according to manufacturer's specification) and thus it is most likely that ZIP14 is cytoprotective by virtue of translocating the only metal, zinc that was experimentally elevated in the extracellular space. The exogenous zinc-mediated protection against LPS was absent in ZIP14 knockdown SPAECs

(Figure 16). Indeed, it appears that in the endothelium ZIP14 is a survival component in LPS signaling.

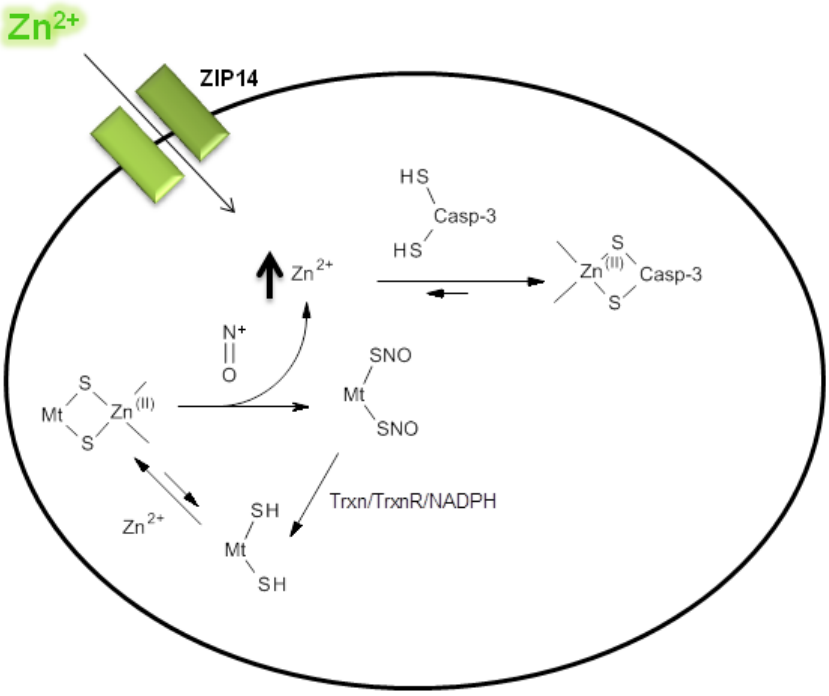


Figure 26. Schematic diagram of potential pathways by which elevation in [Zn]i inhibits LPS -induced apoptosis

Figure 26. ZIP14, a plasma membrane zinc importer, facilitates translocation of zinc from extracellular space to cytosol. Alternatively, NO can S-nitrosate MT leading to an increase in [Zn]i. Elevation in labile zinc is hypothesized to potentially inhibit caspase-3 and block LPS-induced apoptosis. Of note, as previously shown (126), thioredoxin (along with thioredoxin

reductase and NADPH) can denitrosate MT and facilitate its reduction and reincorporation of zinc allowing for recycling and persistence of NO-mediated zinc inhibition of caspase-3.

4.4.2 NO, MT, $[Zn]_i$, and LPS mediated apoptosis in SPAEC

After original reports (10, 88) that NO was capable of causing an increase in detectable labile zinc (including cultured aortic endothelial cells), we were able to identify a critical role for MT in mediating this effect by demonstrating: a) conformational changes in MT via fluorescence resonance energy transfer techniques in live cultured endothelial cells (121) and within the intact pulmonary endothelium of isolated perfused mouse lungs (12); and b) an obligatory and unique role of MT in lung fibroblasts (138) and pulmonary endothelium (137, 147) cultured from MT null mice. In the current study, we extend these observations to another species (sheep) using alternative genetic technology (siRNA) and show that MT is uniquely necessary amongst all zinc bound proteins in accounting for NO-mediated increases in labile zinc (Figure 23). Previously we had coupled NO-MT-Zn^{2+} signaling pathway to aspects of pulmonary endothelial contractility including its contribution to hypoxic pulmonary vasoconstriction (12) via activation of protein kinases and alterations in myosin light chain kinase and phosphatases (11). In the current study, we show for the first time, a definitive link between this pathway (NO-MT-Zn^{2+}) and apoptosis. Our previous report (147) suggested such a link by showing that NO-mediated protection against LPS-induced apoptosis was TPEN sensitive. By selectively genetically reducing expression of MT, the sensor and switch for NO and zinc signaling, and hence removing the NO-mediated increase of labile zinc allowing LPS-induced apoptosis to proceed, a clear linear pathway is established. NO-mediated zinc release is likely to occur either via: a) S-

nitrosation of MT as we have shown in vitro (98); or b) NO activation of protein kinase C and phosphorylation of MT (4). Denitrosation of MT can be facilitated by thioredoxin as we previously reported (141) and lead to reversibility and cycling of these events as shown in figure 26.

In the current study, siRNA suppression of ZIP14 did not affect NO-mediated inhibition of LPS- induced apoptosis (Figure 21). NO, by itself (either via NO donor, SNAP or iNOS derived NO) has been reported to upregulate expression of ZIP14 mRNA (via AP-1) in cultured murine hepatocytes (97). The effect of IL-1β was NO dependent (and MT independent) and resulted in an increase in FluoZin-3 detectable zinc that in turn was sensitive to neutralizing antibodies to ZIP14 (97). We did not detect an increase in mRNA for ZIP14 (Figure 22) in SPAECs exposed to SNAP (up to 1 mM; 6 h), a time interval similar (8 h) to the peak increase in NO-mediated increase in mRNA of ZIP14 in murine hepatocytes (97), suggesting a potential cell specific regulation of ZIP14 expression in response to NO. Changes in mRNA of ZIP14 were dynamic in this latter study (97) and included an early decrease at 0.5 h and a return to baseline by 16 h suggesting that time dependent kinetics could also possible have contributed to differences in the two cell types. We did not assess posttranslational changes in ZIP14 in NO treated SPAEC at either protein level, subcellular location or specific activity and these studies are ultimately critical for a more complete functional determination.

4.4.3 Central Role of $[Zn]_i$ in LPS-mediated apoptosis

A major conclusion from the current study is that elevations in $[Zn]_i$, whether secondary to ZIP-14 mediated translocation of extracellular zinc or NO mediated intracellular release of zinc from

MT, effectively inhibit LPS-mediated apoptosis in SPAEC (Figure 26). In this regard: a) exposure of SPAEC to either 10 μM zinc in extracellular medium (Figures 12 and 15) or 250 μM SNAP (Figure 27) increased FluoZin-3 fluorescence by comparable 25-30% at 6 h; b) in wildtype SPAEC, either 10 μM zinc (Figure 13) or 250 μM SNAP (Figure 20), reduced sensitivity to LPS-induced apoptosis by approximately equivalent 50%; and c) the effect of SNAP was apparent whether zinc was present (Figure 20a) or absent (Figure 20b) in the extracellular space. It was difficult to reproduce precisely equivalent conditions (e.g. 24 h and 100 ng/ml LPS) for experiments involving SNAP and siRNA because: a) in contrast to the SNAP experiments in wildtype cells noted above, the additional 6 h period in SNAP, in combination with interval of siRNA treatment, was associated with additional baseline toxicity that precluded more prolonged (e.g. 24 h) exposure to LPS; and b) readily quantifiable apoptosis was routinely apparent at 4 h after 1 ug/ml of LPS whereas 100 ng/ml LPS had variable effects on apoptosis between subcultures of SPAEC in these shorter (e.g. 4 h) intervals. Accordingly, some comparisons were made, for practical reasons, using 1 μg/ml LPS for 4 h after SNAP (6 h) with a 72 h period of siRNA treatment for either ZIP14 (Figure 21) or MT (Figure 24).

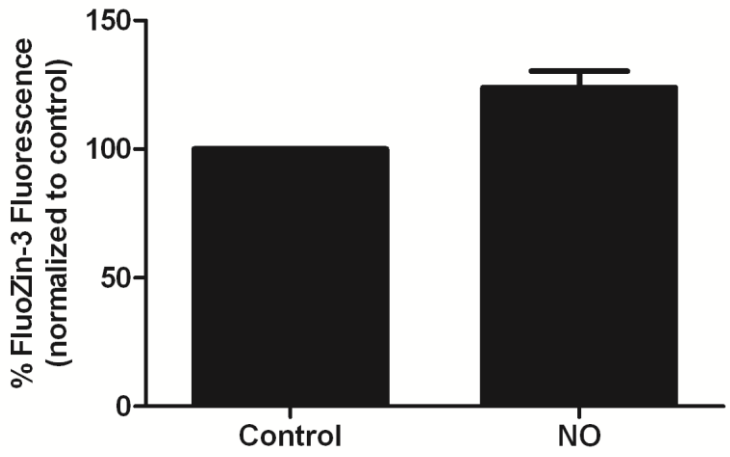


Figure 27. NO (250μM) increases intracellular labile zinc

Figure 27. SPAEC was exposed to NO donor, SNAP (250μM; 6h). Following NO exposure, cells were incubated with FluoZin-3 AM and equal volume of Pluronic F-127 (20 minutes; 37 ^{0}C). Cells were trypsinized, centrifuged and injected into FACS. Data represents mean+SEM of FluoZin-3 fluorescence (% of control) from 5 independent experiments.

Relatively little is known about the contributions of labile zinc and acute lung injury. It is noteworthy that zinc deficiency, secondary to dietary manipulation or alcohol ingestion in rodents is associated with hypersensitivity to polymicrobial sepsis (7, 82) or hyperoxia (149, 150) or macrophage and alveolar epithelial cell dysfunction (74) and the respective phenotypes can be reversed with dietary repletion of zinc. Elevations in labile zinc have also been shown to be antiapoptotic in airway epithelial cells (156, 159). As pulmonary endothelium is a sensitive cell type in acute lung injury and contributes to the maintenance and/or genesis of ARDS from several causes, our results with cytoprotective effects of labile zinc and LPS in SPAEC suggest

that dietary or NO mediated elevations in intracellular zinc may be rational therapies in acute lung injury.

4.4.4 Zinc and caspase-3

The mechanism by which elevations in $[Zn]_i$ inhibit LPS-induced apoptosis remain unclear, in part because numerous pathways in this process may contain presumptive zinc dependent proteins. This becomes even more complex in consideration of the role of elevated $[Zn]_i$ in mediating NO dependent protection as NO, itself, has numerous cellular targets many of which are anti-apoptotic in their own domain (28). From previous reports (122), we focused on caspase-3 and confirmed that zinc was capable of inhibiting its activity directly in vitro (Figure 25) and indirectly in intact cells (Figures 19-20). Although NO can S-nitrosate caspase-3 and inhibit its activity (94), it is apparent from our results that a TPEN-sensitive effect (Figure 19) and requirement of MT (Figure 24a) releasing zinc (Figure 23) in mediating NO protective effect, suggest that direct S-nitrosation of caspase-3 was not likely to account for these collective results (Figure 26). Previous study (122) showed zinc inhibited caspase-3 with an IC50 of 100 nM. Although the binding constant of caspase-3 for zinc is reported to be in the range of 100 nM to 10 μM (84), in an ultra-pure system, 50% inhibition in caspase-3 activity was achieved with 1.7 nM zinc (107). We noted an IC50 of approximately 200 nM zinc (Figure 25) and possible reasons for a lower inhibitor efficiency in our system include: a) presence of the zinc-binding components; phosphates (100 μM) and DTT (1 μM) in the reaction buffer; b) at such a low concentration of zinc (after buffering) the rate of zinc-binding may be too slow to supply caspase-3 with zinc on a biological time scale or c) zinc may not be the sole modulator of caspase-3 activity.

4.5 CONCLUSION

This study adds to accumulating observations underlying the importance of zinc as an intracellular signaling molecule in numerous cellular systems (66) including pulmonary endothelium (11, 12, 153). It is intriguing to speculate that aspects of the role of zinc in regulating LPS-induced apoptosis may provide some insight into the collective experience in which zinc deficiency sensitizes the lung to acute lung injury secondary to hyperoxia (148) or polymicrobial sepsis (82) or alcohol induced alveolar epithelial and macrophage dysfunction (74). Collectively, this study suggests that elevation in labile zinc secondary to zinc release from MT or after influx of zinc via ZIP14 is an important component of the signaling pathway accounting for the inhibition of LPS-induced pulmonary endothelial apoptosis, a contributing factor in LPS-induced lung injury.

4.6 ACKNOWLEDGEMENTS

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5.0 SUMMARY AND FUTURE DIRECTION

5.1 SUMMARY

a) We have reported that LPS mediates a decrease in intracellular labile zinc and this decrease is a critical intracellular signaling component transducing the intrinsic apoptotic effects and transcriptional changes in ZIP14 mRNA in SPAECs.

b) We reported in SPAECs that i) ZIP14 is sensitive to changes in intracellular labile zinc and ii) exogenous zinc mediated protection against LPS-induced apoptosis is dependent upon ZIP14.

c) We reported that NO-mediated protection against LPS-induced apoptosis is dependent upon MT via siRNA to sheep MT isoforms.

d) We further showed that NO-mediated increases in labile zinc are sensitive to MT via siRNA to sheep MT isoforms.

e) We showed that non-toxic dose of zinc chelator, TPEN abrogates NO-mediated protection against LPS-induced apoptosis suggesting that NO-mediated protection against apoptosis is zinc dependent and MT is the source of anti-apoptotic zinc during NO signaling.

f) Elevation of labile $[Zn]_i$ via ZIP14 or NO-MT-Zinc pathway accounts for the decreased sensitivity of SPAECs to LPS.

5.2 NOVELTY AND SIGNIFICANCE

a) Zn^{2+} acts as a second messenger in LPS signaling

b) ZIP14 is a survival factor in LPS signaling

c) Elevation of labile $[Zn]_i$ via ZIP14 or NO-MT-Zn^{2+} signal transduction pathway support a role for the uqibuitous nature of these molecules in sepsis and acute lung injury and make them a rational novel therapeutic target

5.3 FUTURE DIRECTION

To determine the molecular mechanism by which LPS causes a decrease in labile $[Zn]_i$ in SPAECs:

i) Potential changes in activity of zinc exporter, ZnT1 will be measured using Zn^{65} isotope in wild type vs ZnT1 knockdown cells to test whether ZnT1 is the molecular pathway by which LPS decreases labile $[Zn]_{i.}$

ii) X-ray fluorescence microscope will be used to monitor the spatiotemporal movements of labile $[Zn]_i$. in the cell.

iii) LPS-induced changes in labile $[Zn]_i$ and pulmonary endothelial apoptosis will be investigated in intact mouse lung using confocal laser scanning microscope.

iv) The protective role of ZIP14 in LPS-induced apoptosis and lung injury will be confirmed in ZIP14 knockout mouse or via targeted delivery of siRNA to the mouse lung

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